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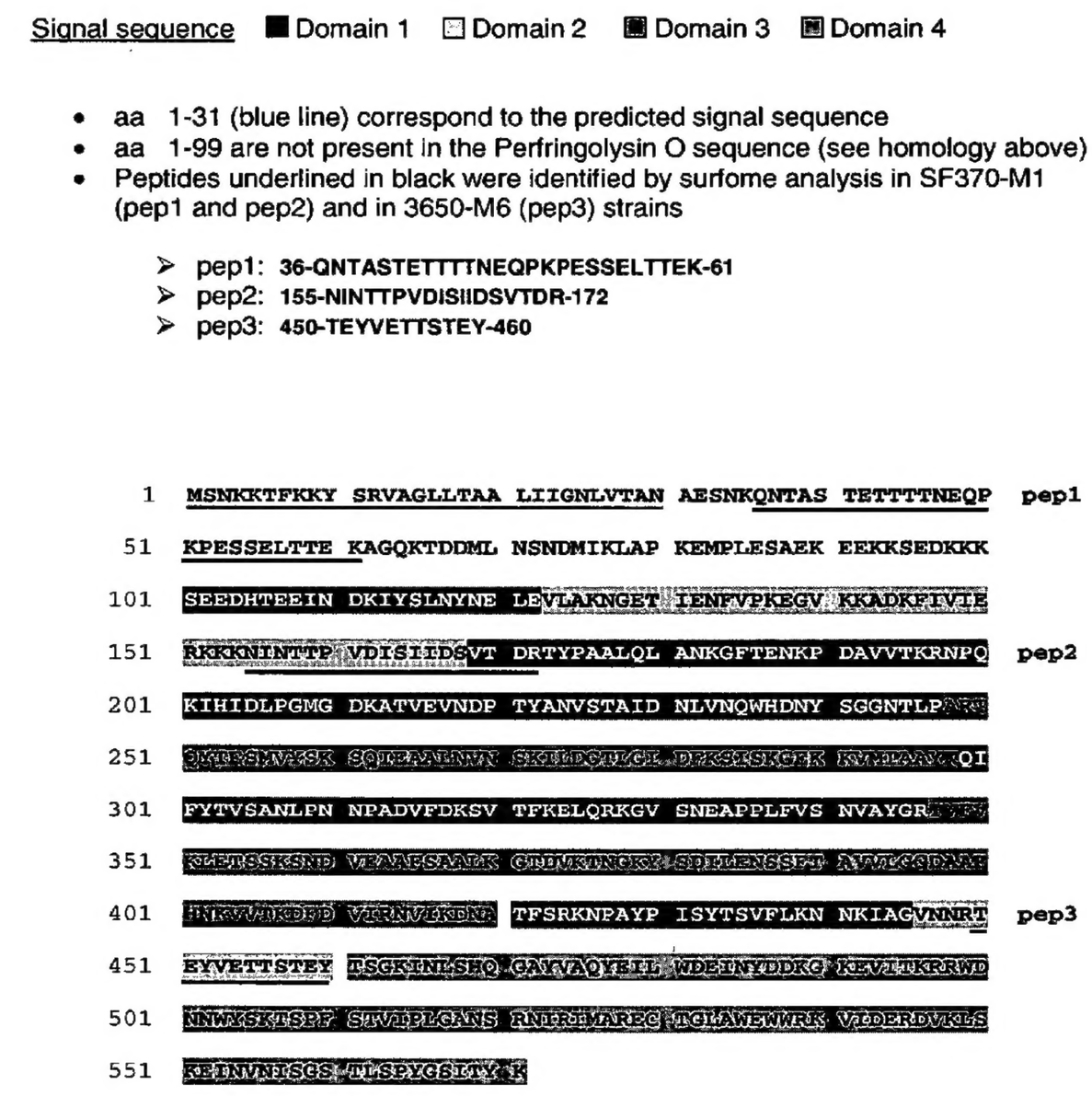
(54) Title: IMMUNOGENIC AND THERAPEUTIC COMPOSITIONS FOR *STREPTOCOCCUS PYOGENES*

(57) Abstract: Compositions for preventing and/or treating S. *pyogenes* infection which comprise one or more active agents. The active agents are SLO antigens, nucleic acid molecules encoding the SLO antigens, and/or antibodies which selectively bind to the SLO antigens.

FIG. 3



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IMMUNOGENIC AND THERAPEUTIC COMPOSITIONS FOR *STREPTOCOCCUS PYOGENES*

[01] This application claims priority to and incorporates by reference provisional application Serial No. 60/855,114 filed October 30, 2006.

FIELD OF THE INVENTION

[02] This invention is in the fields of immunology and vaccinology. In particular, it relates to antigens derived from *Streptococcus pyogenes* and their use in immunization.

BACKGROUND OF THE INVENTION

[03] Streptolysin O (SLO) is an exotoxin produced by *Streptococcus pyogenes* and is inactivated by oxygen (hence the "O" in its name). SLO is oxygen-labile and is a prototype of a prominent family of bacterial toxins known as thiol-activated cytolysins (TACYs). Billington *et al.* 2000 (FEMS Microbiology Letters 18: 197-205).

[04] Thiol-activated cytolysins are toxins produced by a variety of Gram-positive bacteria. These toxins are reversibly inactivated by oxidation and they are characterized by their ability to bind to cholesterol and to promote lysis of cholesterol-containing membranes by binding to cholesterol-containing membranes wherein they polymerize to form pores. Thiol-activated cytolysins are found in more than 20 Gram-positive bacteria and are intimately involved in the pathogenesis of infections by species such as *Arcanobacterium pyogenes* (encoding PLO, or pyolysin), *Clostridium perfringens* (encoding PFO, or perfringolysin), *Listeria monocytogenes* (encoding LLO, or listeriolysin), and *Streptococcus pneumoniae* (encoding PLY or PLN, or pneumolysin).

[05] Sequences of these toxins in different microorganisms are known, *e.g*, Alveolysin (gene alv) from *Bacillus alvei*; Ivanolysin (gene ilo) from *Listeria ivanovii*; Listeriolysin O (gene hlyA) from *Listeria monocytogenes*; Perfringolysin O (theta-toxin) (gene pfo) from *Clostridium perfringens*; Pneumolysin (gene ply) from *Streptococcus pneumoniae*; Seeligeriolysin (gene lso) from *Listeria seeligeri*; and Streptolysin O (gene slo) from

Streptococcus pyogenes. All these proteins contain a single cysteine residue, located in their C-terminal section, which is essential for the binding to cholesterol. This cysteine is located in a highly conserved region that can be used as a signature pattern.

[06] It appears that *Streptococcus pyogenes* uses SLO to translocate an effector protein (*e.g.*, NAD-glycohydrolase) in the host cell which in turn would trigger cytotoxicity. This cytolysin-mediated translocation (CMT) may be the gram-positive equivalent of type III secretion seen in gram-negative pathogens (Cell 2001 104: 143-52).

[07] Unlike many GAS virulence factors, SLO is expressed by almost all GAS isolates, and is encoded by sequences that appear to be highly conserved among distinct M serotypes of GAS. Streptolysin O is highly immunogenic, and determination of the antibody responses engendered to this protein (ASO titer) is often useful in the serodiagnosis of recent infection. Strong antibody responses to SLO have been shown to correlate with the onset of acute rheumatic fever and acute poststreptococcal glomerulonephritis. SLO evokes a protective innate immune response and is a potent inducer of TNFα and IL-1β (see Bricker *et al* 2005).

[08] Because of its immunogenic properties, SLO could be useful in both diagnostic and therapeutic *S. pyogenes* compositions. Unfortunately, SLO is toxic to a wide variety of cell types, including myocardium. There is, therefore, a need in the art for SLO antigens which are not toxic.

BRIEF DESCRIPTION OF THE FIGURES

[09] FIG. 1. Three-dimensional crystal structure of the perfringolysin O monomer from *Clostridium perfringens*.

[10] FIG. 2. BLAST alignment showing GAS25 homology with perfringolysin O from *Clostridium perfringens* (SEQ ID NO:6). GAS25 (SEQ ID NO:5) is the query sequence.

[11] FIG. 3. Prediction of domains in SLO (SEQ ID NO:5) based on the protein sequence homology with *Clostridium perfringens* perfringolysin O. "pep1" is SEQ ID NO:1; "pep2" is SEQ ID NO:2; "pep3" is SEQ ID NO:3.

[12] FIG. 4. Construction of fusion polypeptide containing peptides 2 and 3.

[13] FIG. 5. Cloning and expression of SLO protein fragments as –HIS fusions.

[14] FIG. 6. Cloning and expression of SLO protein fragments as –GST fusions.

[15] FIG. 7. Western blot on total bacterial extracts and purified GST fusion proteins using an anti-GAS25 mouse immune serum.

[16] FIG. 8. Western blot on total bacterial extracts and purified His fusion proteins using an anti-GAS25 mouse immune serum.

[17] FIG. 9. Western blot on purified GST fusion proteins using an anti-GST mouse immune serum.

[18] FIG. 10. Western blot on purified His fusion proteins using an anti-6Xhis commercial monoclonal antibody (Amersham).

[19] FIG. 11. Western Blot with purified GST fusion proteins using different human sera.

[20] FIG. 12. DOT Blot with purified GST fusion proteins using different sera from GAS healthy adults (A: boiled, B: not boiled).

[21] FIG. 13. Western Blot with purified GST fusion proteins using different sera from GAS infected children.

[22] FIG. 14. DOT Blot with boiled (+) and not boiled (-) purified GST fusion proteins using different sera from GAS infected children.

[23] FIG. 15. PAGE analysis of the 6xHIS fusions of three GAS SLO fragments.

[24] FIG. 16. MALDI-TOF analysis of peptide 1 in solution.

[25] FIG. 17. MALDI-TOF analysis of peptide 2+3 in solution.

[26] FIG. 18. MALDI-TOF analysis of peptide 2+3 digested with trypsin.

[27] FIG. 19. MALDI-TOF analysis of peptide 1+2+3 in solution.

[28] FIG. 20. MALDI-TOF analysis of peptide 1+2+3 digested with trypsin.

DETAILED DESCRIPTION OF THE INVENTION

[29] The invention provides compositions for preventing and/or treating *S. pyogenes* infection. These compositions comprise one or more active agents, which are SLO antigens, nucleic acid molecules encoding the SLO antigens, and/or antibodies which selectively bind to the SLO antigens.

SLO antigens

[30] "Streptolysin O (SLO) antigens" according to the invention are immunogenic but not toxic. "Non-toxic" as used herein means that the SLO antigen cannot bind to cholesterol and therefore does not promote lysis of cholesterol-containing membranes. An SLO protein can be rendered non-toxic, for example, by deleting at least the single cysteine residue, located in a highly conserved region in the C-terminal section of SLO that can be used as a signature pattern for thiol-activated cytolysins.

[31] In some embodiments a *Streptococcus pyogenes* streptolysin O (SLO) antigen consists essentially of the amino acid sequence SEQ ID NO:1. In some embodiments an SLO antigen consists essentially of, from N to C terminus, the amino acid sequence SEQ ID NO:2 and the amino acid sequence SEQ ID NO:3 covalently attached to the amino acid sequence SEQ ID NO:2. "Covalently attached" as used herein includes direct covalent linkage as well as linkage via one or more additional amino acids. In other embodiments an SLO antigen consists essentially of, from N to C terminus, the amino acid sequence SEQ ID NO:1; a glycine residue covalently attached to the amino acid sequence SEQ ID NO:1; the amino acid sequence SEQ ID NO:2 covalently attached to the glycine; and the

amino acid sequence SEQ ID NO:3 covalently attached to the amino acid sequence SEQ ID NO:2.

[32] Useful SLO antigens according to the invention also include an amino acid sequence consisting essentially of (1) SEQ ID NO:1; (2) a glycine residue covalently attached to the amino acid sequence SEQ ID NO:1; (3) the amino acid sequence SEQ ID NO:2 covalently attached to the glycine; and (4) the amino acid sequence SEQ ID NO:3 covalently attached to the amino acid sequence SEQ ID NO:2. Still other useful SLO antigens include those consisting essentially of SEQ ID NO:8, SEQ ID NO:10, amino acids 2-82 of SEQ ID NO:10, SEQ ID NO:12, amino acids 4-156 of SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, and SEQ ID NO:18. In some embodiments, the SLO antigen is a monomer.

[33] As there will be variance among SLO antigens between GAS M types and GAS strain isolates, references to the GAS amino acid or polynucleotide sequences of the invention preferably include amino acid or polynucleotide sequences having sequence identity thereto. Preferred amino acid or polynucleotide sequences have 50% or more sequence identity (*e.g.*, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more). Similarly, references to the SLO amino acid or polynucleotide sequences of the invention preferably include fragments of those sequences which retain or encode for the immunological properties of the SLO antigen. Preferred amino acid fragments include at least *n* consecutive amino acids, wherein *n* is 7 or more (*e.g.*, 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50 or more).

Fusion proteins

[34] The SLO antigens used in the invention may be present in the composition as individual separate polypeptides ("peptide 1," "peptide 2," "peptide 3," "peptide 1+2+3," "peptide 2+3"), but there also are embodiments in which at least two (*i.e.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20) antigens are expressed as a single polypeptide chain (a "fusion protein" or "hybrid polypeptide"). Hybrid polypeptides offer two principal advantages. First, a polypeptide that may be unstable or poorly expressed on its

own can be assisted by adding a suitable hybrid partner that overcomes the problem. Second, commercial manufacture is simplified as only one expression and purification need be employed in order to produce two polypeptides which are both antigenically useful.

[35] A hybrid polypeptide may comprise two or more polypeptide sequences. Accordingly, the invention includes a composition comprising a first amino acid sequence and a second amino acid sequence, wherein said first and second amino acid sequences are selected from an SLO antigen or a fragment thereof. Preferably, the first and second amino acid sequences in the hybrid polypeptide comprise different epitopes. In other embodiments, the hybrid polypeptide comprises a first amino acid sequence and a second amino acid sequence, said first amino acid sequence selected from an SLO antigen or a fragment thereof and said second amino acid sequence selected from an SLO antigen or a fragment thereof or from another GAS antigen. Preferably, the first and second amino acid sequences in the hybrid polypeptide comprise different epitopes.

[36] Hybrids consisting of amino acid sequences from two, three, four, five, six, seven, eight, nine, or ten GAS antigens can be constructed. Different hybrid polypeptides may be mixed together in a single formulation. Within such combinations, an SLO antigen may be present in more than one hybrid polypeptide and/or as a non hybrid polypeptide. In some embodiments an antigen is present either as a hybrid or as a non-hybrid, but not as both.

[37] Hybrid polypeptides can be represented by the formula **NH_2-A-{-X-L-}$_n$-B-COOH**, wherein: X is an amino acid sequence of a GAS antigen or a fragment thereof from the first antigen group or the second antigen group; L is an optional linker amino acid sequence; A is an optional N-terminal amino acid sequence; B is an optional C-terminal amino acid sequence; and *n* is 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15.

[38] If a -X- moiety has a leader peptide sequence in its wild-type form, this may be included or omitted in the hybrid protein. In some embodiments, the leader peptides will be deleted except for that of the -X- moiety located at the N-terminus of the hybrid protein

i.e. the leader peptide of X_1 will be retained, but the leader peptides of X_2 ... X_n will be omitted. This is equivalent to deleting all leader peptides and using the leader peptide of X_1 as moiety -A-.

[39] For each *n* instances of {-X-L-}, linker amino acid sequence -L- may be present or absent. For instance, when *n*=2 the hybrid may be NH_2-X_1-L_1-X_2-L_2-COOH, NH_2-X_1-X_2-COOH, NH_2-X_1-L_1-X_2-COOH, NH_2-X_1-X_2-L_2-COOH, *etc.* Linker amino acid sequence(s) -L- will typically be short (*e.g.* 20 or fewer amino acids *i.e.* 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples comprise short peptide sequences which facilitate cloning, poly-glycine linkers (*i.e.* comprising Gly_n where n = 2, 3, 4, 5, 6, 7, 8, 9, 10 or more), and histidine tags (*i.e.* His_n where n = 3, 4, 5, 6, 7, 8, 9, 10 or more). Other suitable linker amino acid sequences will be apparent to those skilled in the art. A useful linker is GSGGGG, with the Gly-Ser dipeptide being formed from a *Bam*HI restriction site, thus aiding cloning and manipulation, and the $(Gly)_4$ tetrapeptide being a typical poly-glycine linker.

[40] -A- is an optional N-terminal amino acid sequence. This will typically be short (*e.g.* 40 or fewer amino acids *i.e.* 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples include leader sequences to direct protein trafficking, or short peptide sequences which facilitate cloning or purification (*e.g.* histidine tags *i.e.* His_n where n = 3, 4, 5, 6, 7, 8, 9, 10 or more). Other suitable N-terminal amino acid sequences will be apparent to those skilled in the art. If X_1 lacks its own N-terminus methionine, -A- is preferably an oligopeptide (*e.g.* with 1, 2, 3, 4, 5, 6, 7 or 8 amino acids) which provides a N-terminus methionine.

[41] -B- is an optional C-terminal amino acid sequence. This will typically be short (*e.g.* 40 or fewer amino acids *i.e.* 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples include sequences to direct protein trafficking, short peptide sequences which facilitate cloning or purification (*e.g.* comprising histidine tags *i.e.* His_n where n = 3, 4, 5, 6, 7, 8, 9, 10 or more), or sequences which enhance protein stability. Other suitable C-terminal amino acid sequences will be apparent to those skilled in the art.

[42] Most preferably, n is 2 or 3.

[43] The fusion constructs of the invention may include a combination of two or more SLO antigens. Preferred combinations include fusions with a GAS40 or GAS57 antigen.

GAS40

[44] GAS40 antigens are particularly useful in compositions of the invention because GAS40 proteins are highly conserved both in many M types and in multiple strains of these M types (see WO 2006/042027). GAS40 proteins are described in detail in WO 2005/032582. GAS40 consistently provides protection in the animal model of systemic immunization and challenge and induction of bactericidal antibodies. GAS40 is an extremely highly conserved protein and appears to be exposed on the surface of most M serotypes (the only exception observed thus far is the M3 serotype).

[45] Amino acid sequences of a number of GAS40 proteins from various M strains are contained in GenBank and have accession numbers GI:13621545 and GI:15674449 (M1); accession number GI: 21909733 (M3), and accession number GI:19745402 (M18). GAS40 proteins also are known as "Spy0269" (M1), "SpyM3_0197" (M3), "SpyM18_0256" (M18) and "prgA."

[46] A GAS40 protein typically contains a leader peptide sequence (*e.g.*, amino acids 1 – 26 of SEQ ID NO:19), a first coiled-coil region (*e.g.*, amino acids 58 – 261 of SEQ ID NO:19), a second coiled coil region (*e.g.*, amino acids 556 – 733 of SEQ ID NO:19), a leucine zipper region (*e.g.*, amino acids 673 – 701 of SEQ ID NO:19) and a transmembrane region (*e.g.*, amino acids 855 – 866 of SEQ ID NO:19).

[47] Preferred GAS40 proteins for use with the invention comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO:19; and/or (b) which is a fragment of at least n consecutive amino acids of SEQ ID NO:19, wherein n is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200 or more). These GAS40 proteins include variants (*e.g.* allelic variants, homologs,

orthologs, paralogs, mutants, etc.) of SEQ ID NO:19. Preferred fragments of a GAS40 protein lack one or more amino acids (*e.g.*,1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.*,1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of the GAS40 protein. In one embodiment, the leader sequence is removed. In another embodiment, the transmembrane region is removed. Other fragments may omit one or more other domains of the GAS40 protein.

[48] The coiled-coil regions of GAS40 are likely involved in the formation of oligomers such as dimers or trimers. Such oligomers could be homomers (containing two or more GAS40 proteins oligomerized together) or heteromers (containing one or more additional GAS proteins oligomerized with GAS40). Alternatively, two coiled-coil regions may interact together within the GAS40 protein to form oligomeric reactions between the first and second coiled-coil regions. Thus, in some embodiments the GAS40 antigen is in the form of an oligomer. Some oligomers comprise two more GAS40 antigens. Other oligomers comprise a GAS40 antigen oligomerized to a second GAS antigen.

GAS57

[49] GAS57 corresponds to M1 GenBank accession numbers GI:13621655 and GI:15674549, to M3 GenBank accession number GI: 21909834, to M18 GenBank accession number GI: 19745560 and is also referred to as 'Spy0416' (M1), 'SpyM3_0298' (M3), 'SpyM18_0464' (M18) and 'prtS.' GAS57 has also been identified as a putative cell envelope proteinase. The amino acid sequence of GAS57 of an M1 strain is set forth in the sequence listing as SEQ ID NO:20.

[50] Preferred GAS57 proteins for use with the invention comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO:20; and/or (b) which is a fragment of at least n consecutive amino acids of SEQ ID NO:20, wherein n is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200 or more). These GAS57 proteins include variants (*e.g.* allelic variants, homologs, orthologs, paralogs, mutants, etc.) of SEQ ID NO:20. Preferred fragments of (b) comprise

an epitope from SEQ ID NO:20. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO:20. For example, in one embodiment, amino acids 1-33 are removed. In another example, amino acids 1614-1647 or SEQ ID NO:20 are removed. Other fragments omit one or more domains of the protein (*e.g.* omission of a signal peptide, of a cytoplasmic domain, of a transmembrane domain, or of an extracellular domain).

Nucleic Acid Molecules

[51] The invention includes nucleic acid molecules which encode SLO antigens. The invention also includes nucleic acid molecules comprising nucleotide sequences having at least 50% sequence identity to such molecules. Depending on the particular sequence, the degree of sequence identity is preferably greater than 50% (*e.g.*, 60%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more). Identity between nucleotide sequences is preferably determined by the Smith-Waterman homology search algorithm as implemented in the MPSRCH program (Oxford Molecular), using an affine gap search with parameters gap open penalty = 12 and gap extension penalty = 1.

[52] The invention also provides nucleic acid molecules which can hybridize to these molecules. Hybridization reactions can be performed under conditions of different "stringency." Conditions which increase stringency of a hybridization reaction are widely known and published in the art. See, *e.g.*, page 7.52 of Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 1989. Examples of relevant conditions include (in order of increasing stringency): incubation temperatures of 25°C, 37 °C, 50 °C, 55 °C, and 68 °C; buffer concentrations of 10X SSC, 6X SSC, 1X SSC, and 0.1X SSC (where SSC is 0.15 M NaCl and 15 mM citrate buffer) and their equivalents using other buffer systems; formamide concentrations of 0%, 25%, 50%, and 75%; incubation times from 5 minutes to 24 hours; 1, 2, or more washing steps; wash incubation times of 1, 2, or 15 minutes; and wash solutions of 6X SSC, 1X SSC, 0.1X SSC, or de-ionized

water. Hybridization techniques and their optimization are well known in the art. See, *e.g.*, Sambrook, 1989; Ausubel *et al.*, eds., Short Protocols in Molecular Biology, 4th ed., 1999; U.S. Patent 5,707,829; Ausubel *et al.*, eds., Current Protocols in Molecular Biology, Supplement 30, 1987.

[53] In some embodiments, nucleic acid molecules of the invention hybridize to a target under low stringency conditions; in other embodiments, nucleic acid molecules of the invention hybridize under intermediate stringency conditions; in preferred embodiments, nucleic acid molecules of the invention hybridize under high stringency conditions. An example of a low stringency hybridization condition is 50^{oC} and 10X SSC. An example of an intermediate stringency hybridization condition is 55°C and 1X SSC. An example of a high stringency hybridization condition is 68°C and 0.1X SSC.

[54] Nucleic acid molecules comprising fragments of these sequences are also included in the invention. These comprise at least n consecutive nucleotides of these sequences and, depending on the particular sequence, n is 10 or more (*e.g.*, 12, 14, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, or more).

[55] Nucleic acids (and polypeptides) of the invention may include sequences which:

(a) are identical (*i.e.*, 100% identical) to the sequences disclosed in the sequence listing;

(b) share sequence identity with the sequences disclosed in the sequence listing;

(c) have 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 single nucleotide or amino acid alterations (deletions, insertions, substitutions), which may be at separate locations or may be contiguous, as compared to the sequences of (a) or (b); and,

d) when aligned with a particular sequence from the sequence listing using a pairwise alignment algorithm, a moving window of x monomers (amino acids or nucleotides) moving from start (N-terminus or 5′) to end (C-terminus or 3′), such that for an alignment that extends to p monomers (where p>x) there are p-x+1 such windows,

each window has at least x·y identical aligned monomers, where: x is selected from 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200; y is selected from 0.50, 0.60, 0.70, 0.75, 0.80, 0.85, 0.90, 0.91, 0.92, 0.93, 0.94, 0.95, 0.96, 0.97, 0.98, 0.99; and if x·y is not an integer then it is rounded up to the nearest integer. The preferred pairwise alignment algorithm is the Needleman-Wunsch global alignment algorithm [Needleman & Wunsch (1970) J. Mol. Biol. 48, 443-453], using default parameters (*e.g.*, with Gap opening penalty = 10.0, and with Gap extension penalty = 0.5, using the EBLOSUM62 scoring matrix). This algorithm is conveniently implemented in the needle tool in the EMBOSS package [Rice *et al.* (2000) Trends Genet. 16:276-277].

[56] The nucleic acids and polypeptides of the invention may additionally have further sequences to the N-terminus/5′ and/or C-terminus/3′ of these sequences (a) to (d).

Antibodies

[57] Antibodies can be generated to bind specifically to an SLO antigen of the invention. The term "antibody" includes intact immunoglobulin molecules, as well as fragments thereof which are capable of binding an antigen. These include hybrid (chimeric) antibody molecules (*e.g.*, Winter *et al.*, Nature 349, 293-99, 1991; U.S. Patent 4,816,567); F(ab′)2 and F(ab) fragments and Fv molecules; non-covalent heterodimers (*e.g.*, Inbar *et al.*, Proc. Natl. Acad. Sci. U.S.A. 69, 2659-62, 1972; Ehrlich *et al.*, Biochem 19, 4091-96, 1980); single-chain Fv molecules (sFv) (*e.g.*, Huston *et al.*, Proc. Natl. Acad. Sci. U.S.A. 85, 5897-83, 1988); dimeric and trimeric antibody fragment constructs; minibodies (*e.g.*, Pack *et al.*, Biochem 31, 1579-84, 1992; Cumber *et al.*, J. Immunology 149B, 120-26, 1992); humanized antibody molecules (*e.g.*, Riechmann *et al.*, Nature 332, 323-27, 1988; Verhoeyan *et al.*, Science 239, 1534-36, 1988; and U.K. Patent Publication No. GB 2,276,169, published 21 September 1994); and any functional fragments obtained from such molecules, as well as antibodies obtained through non-conventional processes such as phage display. Preferably, the antibodies are monoclonal antibodies. Methods of obtaining monoclonal antibodies are well known in the art.

[58] Typically, at least 6, 7, 8, 10, or 12 contiguous amino acids are required to form an epitope. However, epitopes which involve non-contiguous amino acids may require more, *e.g.*, at least 15, 25, or 50 amino acids. Various immunoassays (*e.g.*, Western blots, ELISAs, radioimmunoassays, immunohistochemical assays, immunoprecipitations, or other immunochemical assays known in the art) can be used to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays are well known in the art. Such immunoassays typically involve the measurement of complex formation between an immunogen and an antibody which specifically binds to the immunogen. A preparation of antibodies which specifically bind to a particular antigen typically provides a detection signal at least 5-, 10-, or 20-fold higher than a detection signal provided with other proteins when used in an immunochemical assay. Preferably, the antibodies do not detect other proteins in immunochemical assays and can immunoprecipitate the particular antigen from solution.

Generation of antibodies

[59] SLO antigens or non-SLO polypeptide antigens (described below) can be used to immunize a mammal, such as a mouse, rat, rabbit, guinea pig, monkey, or human, to produce polyclonal antibodies. If desired, an antigen can be conjugated to a carrier protein, such as bovine serum albumin, thyroglobulin, and keyhole limpet hemocyanin. Depending on the host species, various adjuvants can be used to increase the immunological response. Such adjuvants include, but are not limited to, Freund's adjuvant, mineral gels (*e.g.*, aluminum hydroxide), and surface active substances (*e.g.* lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol). Among adjuvants used in humans, BCG (bacilli Calmette-Guerin) and Corynebacterium parvum are especially useful.

[60] Monoclonal antibodies which specifically bind to an antigen can be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These techniques include, but are not limited to, the hybridoma technique, the human B cell hybridoma technique, and the EBV hybridoma technique (Kohler *et al.*, Nature 256, 495 497, 1985; Kozbor *et al.*, J. Immunol. Methods 81, 31 42,

1985; Cote *et al.*, Proc. Natl. Acad. Sci. 80, 2026 2030, 1983; Cole *et al.*, Mol. Cell Biol. 62, 109 120, 1984).

[61] In addition, techniques developed for the production of "chimeric antibodies," the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity, can be used (Morrison *et al.*, Proc. Natl. Acad. Sci. 81, 6851 6855, 1984; Neuberger *et al.*, Nature 312, 604 608, 1984; Takeda *et al.*, Nature 314, 452 454, 1985). Monoclonal and other antibodies also can be "humanized" to prevent a patient from mounting an immune response against the antibody when it is used therapeutically. Such antibodies may be sufficiently similar in sequence to human antibodies to be used directly in therapy or may require alteration of a few key residues. Sequence differences between rodent antibodies and human sequences can be minimized by replacing residues which differ from those in the human sequences by site directed mutagenesis of individual residues or by grating of entire complementarity determining regions.

[62] Alternatively, humanized antibodies can be produced using recombinant methods, as described below. Antibodies which specifically bind to a particular antigen can contain antigen binding sites which are either partially or fully humanized, as disclosed in U.S. 5,565,332.

[63] Alternatively, techniques described for the production of single chain antibodies can be adapted using methods known in the art to produce single chain antibodies which specifically bind to a particular antigen. Antibodies with related specificity, but of distinct idiotypic composition, can be generated by chain shuffling from random combinatorial immunoglobin libraries (Burton, Proc. Natl. Acad. Sci. 88, 11120 23, 1991).

[64] Single-chain antibodies also can be constructed using a DNA amplification method, such as PCR, using hybridoma cDNA as a template (Thirion *et al.*, 1996, Eur. J. Cancer Prev. 5, 507-11). Single-chain antibodies can be mono- or bispecific, and can be bivalent or tetravalent. Construction of tetravalent, bispecific single-chain antibodies is taught, for

example, in Coloma & Morrison, 1997, Nat. Biotechnol. 15, 159-63. Construction of bivalent, bispecific single-chain antibodies is taught in Mallender & Voss, 1994, J. Biol. Chem. 269, 199-206.

[65] A nucleotide sequence encoding a single-chain antibody can be constructed using manual or automated nucleotide synthesis, cloned into an expression construct using standard recombinant DNA methods, and introduced into a cell to express the coding sequence, as described below. Alternatively, single-chain antibodies can be produced directly using, for example, filamentous phage technology (Verhaar *et al.*, 1995, Int. J. Cancer 61, 497-501; Nicholls *et al.*, 1993, J. Immunol. Meth. 165, 81-91).

[66] Antibodies which specifically bind to a particular antigen also can be produced by inducing in vivo production in the lymphocyte population or by screening immunoglobulin libraries or panels of highly specific binding reagents as disclosed in the literature (Orlandi *et al.*, Proc. Natl. Acad. Sci. 86, 3833 3837, 1989; Winter *et al.*, Nature 349, 293 299, 1991).

[67] Chimeric antibodies can be constructed as disclosed in WO 93/03151. Binding proteins which are derived from immunoglobulins and which are multivalent and multispecific, such as the "diabodies" described in WO 94/13804, also can be prepared.

[68] Antibodies can be purified by methods well known in the art. For example, antibodies can be affinity purified by passage over a column to which the relevant antigen is bound. The bound antibodies can then be eluted from the column using a buffer with a high salt concentration.

Production of polypeptide antigens

Recombinant production of polypeptides

[69] Any nucleotide sequence which encodes a particular antigen can be used to produce that antigen recombinantly. If desired, an antibody can be produced recombinantly once its amino acid sequence is known.

[70] Examples of sequences which can be used to produce SLO antigens of the invention are shown in FIGS. 5 and 6. Nucleic acid molecules encoding SLO can be isolated from the appropriate *S. pyogenes* bacterium using standard nucleic acid purification techniques or can be synthesized using an amplification technique, such as the polymerase chain reaction (PCR), or by using an automatic synthesizer. Methods for isolating nucleic acids are routine and are known in the art. Any such technique for obtaining nucleic acid molecules can be used to obtain a nucleic acid molecule which encodes a particular antigen. Sequences encoding a particular antigen or antibody can be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers *et al.*, Nucl. Acids Res. Symp. Ser. 215 223, 1980; Horn *et al.* Nucl. Acids Res. Symp. Ser. 225 232, 1980).

[71] cDNA molecules can be made with standard molecular biology techniques, using mRNA as a template. cDNA molecules can thereafter be replicated using molecular biology techniques well known in the art. An amplification technique, such as PCR, can be used to obtain additional copies of polynucleotides of the invention, using either genomic DNA or cDNA as a template.

[72] If desired, nucleotide sequences can be engineered using methods generally known in the art to alter antigen-encoding sequences for a variety of reasons, including but not limited to, alterations which modify the cloning, processing, and/or expression of the polypeptide or mRNA product. DNA shuffling by random fragmentation and PCR reassembly of gene fragments and synthetic oligonucleotides can be used to engineer the nucleotide sequences. For example, site directed mutagenesis can be used to insert new restriction sites, alter glycosylation patterns, change codon preference, produce splice variants, introduce mutations, and so forth.

[73] Sequence modifications, such as the addition of a purification tag sequence or codon optimization, can be used to facilitate expression. For example, the N-terminal leader sequence may be replaced with a sequence encoding for a tag protein such as polyhistidine ("HIS") or glutathione S-transferase ("GST"). Such tag proteins may be used to facilitate purification, detection, and stability of the expressed protein. Codons

preferred by a particular prokaryotic or eukaryotic host can be selected to increase the rate of protein expression or to produce an RNA transcript having desirable properties, such as a half life which is longer than that of a transcript generated from the naturally occurring sequence. These methods are well known in the art and are further described in WO05/032582.

Expression vectors

[74] A nucleic acid molecule which encodes an antigen or antibody can be inserted into an expression vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. Methods which are well known to those skilled in the art can be used to construct expression vectors containing coding sequences and appropriate transcriptional and translational control elements. These methods include in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination.

Host cells

[75] The heterologous host can be prokaryotic or eukaryotic. *E. coli* is a preferred host cell, but other suitable hosts include *Lactococcus lactis, Lactococcus cremoris, Bacillus subtilis, Vibrio cholerae, Salmonella typhi, Salmonella typhimurium, Neisseria lactamica, Neisseria cinerea, Mycobacteria* (*e.g., M. tuberculosis*), yeasts, etc.

[76] A host cell strain can be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed polypeptide in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Post translational processing which cleaves a "prepro" form of the polypeptide also can be used to facilitate correct insertion, folding and/or function. Different host cells which have specific cellular machinery and characteristic mechanisms for post translational activities are available from the American Type Culture Collection (ATCC; 10801 University Boulevard, Manassas, VA 20110-2209) and can be chosen to ensure the correct modification and processing of a foreign protein. See WO 01/98340.

[77] Expression constructs can be introduced into host cells using well-established techniques which include, but are not limited to, transferrin-polycation-mediated DNA transfer, transfection with naked or encapsulated nucleic acids, liposome-mediated cellular fusion, intracellular transportation of DNA-coated latex beads, protoplast fusion, viral infection, electroporation, "gene gun" methods, and DEAE- or calcium phosphate-mediated transfection.

[78] Host cells transformed with expression vectors can be cultured under conditions suitable for the expression and recovery of the protein from cell culture. The protein produced by a transformed cell can be secreted or contained intracellularly depending on the nucleotide sequence and/or the expression vector used. Those of skill in the art understand that expression vectors can be designed to contain signal sequences which direct secretion of soluble antigens through a prokaryotic or eukaryotic cell membrane.

Purification

[79] Antigens used in the invention can be isolated from the appropriate *Streptococcus pyogenes* bacterium or from an engineered host cell. A purified polypeptide antigen is separated from other components in the cell, such as proteins, carbohydrates, or lipids, using methods well-known in the art. Such methods include, but are not limited to, size exclusion chromatography, ammonium sulfate fractionation, ion exchange chromatography, affinity chromatography, and preparative gel electrophoresis. A preparation of purified polypeptide antigens is at least 80% pure; preferably, the preparations are 90%, 95%, or 99% pure. Purity of the preparations can be assessed by any means known in the art, such as SDS-polyacrylamide gel electrophoresis. Where appropriate, polypeptide antigens can be solubilized, for example, with urea.

Chemical synthesis

[80] SLO antigens, as well as other antigens used in compositions of the invention, can be synthesized, for example, using solid phase techniques. See, *e.g.*, Merrifield, J. Am. Chem. Soc. 85, 2149 54, 1963; Roberge *et al.*, Science 269, 202 04, 1995. Protein synthesis can be performed using manual techniques or by automation. Automated

synthesis can be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer). Optionally, fragments of an SLO antigen can be separately synthesized and combined using chemical methods to produce a full-length molecule.

[81] Nucleic acid molecules which encode antibodies or polypeptide antigens can be synthesized by conventional methodology, such as the phosphate triester method (Hunkapiller, M. *et al.* (1984), Nature 310: 105-111) or by the chemical synthesis of nucleic acids (Grantham, R. *et al.* (1981), Nucleic Acids Res. 9: r43-r74).

Immunogenic, Diagnostic, and Therapeutic Compositions

[82] The invention also provides compositions for use as medicaments (*e.g.*, as immunogenic compositions or vaccines) or as diagnostic reagents for detecting a GAS infection in a host subject. It also provides the use of the compositions in the manufacture of: (i) a medicament for treating or preventing infection due to GAS bacteria; (ii) a diagnostic reagent for detecting the presence of GAS bacteria or of antibodies raised against GAS bacteria; and/or (iii) a reagent which can raise antibodies against GAS bacteria.

[83] For example, SLO antigens or nucleic acids encoding the antigens can be used in the manufacture of a diagnostic reagent for detecting the presence of a GAS infection or for detecting antibodies raised against GAS bacteria, or in the manufacture of a reagent which can raise antibodies against GAS bacteria. Nucleic acids encoding SLO antigens can be detected by contacting a nucleic acid probe with a biological sample under hybridizing conditions to form duplexes and detecting the duplexes as is known in the art. An SLO antigen can be detected using antibodies which specifically bind to the SLO antigen. Similarly, antibodies to SLO antigens can be used to detect SLO antigens by contacting a biological sample under conditions suitable for the formation of antibody-antigen complexes and detecting any complexes formed. The invention also provides kits comprising reagents suitable for use these methods.

Therapeutic compositions

[84] Compositions of the invention are useful for preventing and/or treating *S. pyogenes* infection. Compositions containing SLO antigens are preferably immunogenic compositions, and are more preferably vaccine compositions. The pH of such compositions preferably is between 6 and 8, preferably about 7. The pH can be maintained by the use of a buffer. The composition can be sterile and/or pyrogen free. The composition can be isotonic with respect to humans.

[85] Vaccines according to the invention may be used either prophylactically or therapeutically, but will typically be prophylactic. Accordingly, the invention includes a method for the therapeutic or prophylactic treatment of a *Streptococcus pyogenes* infection. The animal is preferably a mammal, most preferably a human. The methods involve administering to the animal a therapeutic or prophylactic amount of the immunogenic compositions of the invention.

[86] Some compositions of the invention comprise a polypeptide SLO antigen as described herein. Other compositions of the invention comprise a nucleic acid molecule which encodes the SLO antigen(s) and, optionally, other antigens which can be included in the composition (see below). See, *e.g.*, Robinson & Torres (1997) Seminars in Immunology 9:271-283; Donnelly *et al.* (1997) Ann. Rev Immunol 15:617-648; Scott-Taylor & Dalgleish (2000) Expert Opin Investig Drugs 9:471-480; Apostolopoulos & Plebanski (2000) Curr Opin Mol Ther 2:441-447; Ilan (1999) Curr Opin Mol Ther 1:116-120; Dubensky *et al.* (2000) Mol Med 6:723-732; Robinson & Pertmer (2000) Adv Virus Res 55:1-74; Donnelly *et al.* (2000) Am J Respir Crit Care Med 162(4 Pt 2):S190-193; Davis (1999) Mt. Sinai J. Med. 66:84-90. Typically the nucleic acid molecule is a DNA molecule, *e.g.*, in the form of a plasmid.

[87] Compositions for treating *S. pyogenes* infections comprise at least one antibody which specifically binds to an SLO antigen and, optionally, an antibody which specifically binds to a non-SLO antigen. Some compositions of the invention are immunogenic and comprise one or more polypeptide antigens, while other immunogenic compositions

comprise nucleic acid molecules which encode one or more antigens. See, *e.g.*, Robinson & Torres (1997) Seminars in Immunology 9:271-283; Donnelly *et al.* (1997) Ann. Rev Immunol 15:617-648; Scott-Taylor & Dalgleish (2000) Expert Opin Investig Drugs 9:471-480; Apostolopoulos & Plebanski (2000) Curr Opin Mol Ther 2:441-447; Ilan (1999) Curr Opin Mol Ther 1:116-120; Dubensky *et al.* (2000) Mol Med 6:723-732; Robinson & Pertmer (2000) Adv Virus Res 55:1-74; Donnelly *et al.* (2000) Am J Respir Crit Care Med 162(4 Pt 2):S190-193Davis (1999) Mt. Sinai J. Med. 66:84-90. Typically the nucleic acid molecule is a DNA molecule, *e.g.*, in the form of a plasmid.

[88] In some embodiments, compositions of the invention can include one or more additional active agents. Such agents include, but are not limited to, (a) another SLO antigen of the invention, (b) a polypeptide antigen which is useful in a pediatric vaccine, (c) a polypeptide antigen which is useful in a vaccine for elderly or immunocompromised individuals, (d) a nucleic acid molecule encoding (a)-(c), and an antibody which specifically binds to (a)-(c).

Additional antigens

[89] Compositions of the invention may be administered in conjunction with one or more antigens for use in therapeutic, prophylactic, or diagnostic methods of the present invention. Preferred antigens include those listed below. Additionally, the compositions of the present invention may be used to treat or prevent infections caused by any of the below-listed pathogens. In addition to combination with the antigens described below, the compositions of the invention may also be combined with an adjuvant as described herein.

[90] Antigens for use with the invention include, but are not limited to, one or more of the following antigens set forth below, or antigens derived from one or more of the pathogens set forth below:

A. Bacterial Antigens

[91] Bacterial antigens suitable for use in the invention include proteins, polysaccharides, lipopolysaccharides, and outer membrane vesicles which may be isolated, purified or derived from a bacteria. In addition, bacterial antigens may include bacterial lysates and inactivated bacteria formulations. Bacteria antigens may be produced by recombinant expression. Bacterial antigens preferably include epitopes which are exposed on the surface of the bacteria during at least one stage of its life cycle. Bacterial antigens are preferably conserved across multiple serotypes. Bacterial antigens include antigens derived from one or more of the bacteria set forth below as well as the specific antigens examples identified below.

[92] *Neisseria meningitides*: *Meningitides* antigens may include proteins (such as those identified in References 1 – 7), saccharides (including a polysaccharide, oligosaccharide or lipopolysaccharide), or outer-membrane vesicles (References 8, 9, 10, 11) purified or derived from *N. meningitides* serogroup such as A, C, W135, Y, and/or B. *Meningitides* protein antigens may be selected from adhesions, autotransporters, toxins, Fe acquisition proteins, and membrane associated proteins (preferably integral outer membrane protein).

[93] *Streptococcus pneumoniae*: *Streptococcus pneumoniae* antigens may include a saccharide (including a polysaccharide or an oligosaccharide) and/or protein from *Streptococcus pneumoniae*. Saccharide antigens may be selected from serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F. Protein antigens may be selected from a protein identified in WO 98/18931, WO 98/18930, US Patent No. 6,699,703, US Patent No. 6,800,744, WO 97/43303, and WO 97/37026. *Streptococcus pneumoniae* proteins may be selected from the Poly Histidine Triad family (PhtX), the Choline Binding Protein family (CbpX), CbpX truncates, LytX family, LytX truncates, CbpX truncate-LytX truncate chimeric proteins, pneumolysin (Ply), PspA, PsaA, Sp128, Sp101, Sp130, Sp125 or Sp133.

[94] *Streptococcus pyogenes* (Group A Streptococcus): Group A *Streptococcus* antigens may include a protein identified in WO 02/34771 or WO 2005/032582 (including GAS 40),

fusions of fragments of GAS M proteins (including those described in WO 02/094851, and Dale, Vaccine (1999) 17:193-200, and Dale, Vaccine 14(10): 944-948), fibronectin binding protein (Sfb1), Streptococcal heme-associated protein (Shp), and Streptolysin S (SagA).

[95] *Moraxella catarrhalis*: *Moraxella* antigens include antigens identified in WO 02/18595 and WO 99/58562, outer membrane protein antigens (HMW-OMP), C-antigen, and/or LPS.

[96] *Bordetella pertussis*: Pertussis antigens include petussis holotoxin (PT) and filamentous haemagglutinin (FHA) from *B. pertussis*, optionally also combination with pertactin and/or agglutinogens 2 and 3 antigen.

[97] *Staphylococcus aureus*: *Staphylococcus aureus* antigens include *S. aureus* type 5 and 8 capsular polysaccharides optionally conjugated to nontoxic recombinant *Pseudomonas aeruginosa* exotoxin A, such as StaphVAX™, or antigens derived from surface proteins, invasins (leukocidin, kinases, hyaluronidase), surface factors that inhibit phagocytic engulfment (capsule, Protein A), carotenoids, catalase production, Protein A, coagulase, clotting factor, and/or membrane-damaging toxins (optionally detoxified) that lyse eukaryotic cell membranes (hemolysins, leukotoxin, leukocidin).

[98] *Staphylococcus epidermis*: *S. epidermidis* antigens include slime-associated antigen (SAA).

[99] *Clostridium tetani* (Tetanus): Tetanus antigens include tetanus toxoid (TT), preferably used as a carrier protein in conjunction/conjugated with the compositions of the present invention.

[100] *Cornynebacterium diphtheriae* (Diphtheria): Diphtheria antigens include diphtheria toxin, preferably detoxified, such as CRM197. Additionally antigens capable of modulating, inhibiting or associated with ADP ribosylation are contemplated for combination/co-administration/conjugation with the compositions of the present invention. The diphtheria toxoids may be used as carrier proteins.

[101] *Haemophilus influenzae B* (Hib): Hib antigens include a Hib saccharide antigen.

[102] *Pseudomonas aeruginosa*: *Pseudomonas* antigens include endotoxin A, Wzz protein, P. aeruginosa LPS, more particularly LPS isolated from PAO1 (O5 serotype), and/or Outer Membrane Proteins, including Outer Membrane Proteins F (OprF) (Infect Immun. 2001 May; 69(5): 3510-3515).

[103] *Legionella pneumophila.* Bacterial antigens may be derived from *Legionella pneumophila.*

[104] *Streptococcus agalactiae* (Group B Streptococcus): Group B *Streptococcus* antigens include a protein or saccharide antigen identified in WO 02/34771, WO 03/093306, WO 04/041157, or WO 2005/002619 (including proteins GBS 80, GBS 104, GBS 276 and GBS 322, and including saccharide antigens derived from serotypes Ia, Ib, Ia/c, II, III, IV, V, VI, VII and VIII).

[105] *Neiserria gonorrhoeae*: *Gonorrhoeae* antigens include Por (or porin) protein, such as PorB (see Zhu *et al.*, Vaccine (2004) 22:660 – 669), a transferring binding protein, such as TbpA and TbpB (See Price *et al.*, Infection and Immunity (2004) 71(1):277 – 283), a opacity protein (such as Opa), a reduction-modifiable protein (Rmp), and outer membrane vesicle (OMV) preparations (see Plante *et al.*, J Infectious Disease (2000) 182:848 – 855), also see *e.g.* WO99/24578, WO99/36544, WO99/57280, WO02/079243).

[106] *Chlamydia trachomatis*: *Chlamydia trachomatis* antigens include antigens derived from serotypes A, B, Ba and C (agents of trachoma, a cause of blindness), serotypes L1, L2 & L3 (associated with *Lymphogranuloma venereum*), and serotypes, D-K. Chlamydia trachomas antigens may also include an antigen identified in WO 00/37494, WO 03/049762, WO 03/068811, or WO 05/002619, including PepA (CT045), LcrE (CT089), ArtJ (CT381), DnaK (CT396), CT398, OmpH-like (CT242), L7/L12 (CT316), OmcA (CT444), AtosS (CT467), CT547, Eno (CT587), HrtA (CT823), and MurG (CT761).

[107] *Treponema pallidum* (Syphilis): Syphilis antigens include TmpA antigen.

[108] *Haemophilus ducreyi* (causing chancroid): Ducreyi antigens include outer membrane protein (DsrA).

[109] *Enterococcus faecalis* or *Enterococcus faecium*: Antigens include a trisaccharide repeat or other *Enterococcus* derived antigens provided in US Patent No. 6,756,361.

[110] *Helicobacter pylori*: *H. pylori* antigens include Cag, Vac, Nap, HopX, HopY and/or urease antigen.

[111] *Staphylococcus saprophyticus*: Antigens include the 160 kDa hemagglutinin of *S. saprophyticus* antigen.

[112] *Yersinia enterocolitica* antigens include LPS (Infect Immun. 2002 August; 70(8): 4414).

[113] *E. coli*: *E. coli* antigens may be derived from enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAggEC), diffusely adhering *E. coli* (DAEC), enteropathogenic *E. coli* (EPEC), and/or enterohemorrhagic *E. coli* (EHEC).

[114] *Bacillus anthracis* (anthrax): *B. anthracis* antigens are optionally detoxified and may be selected from A-components (lethal factor (LF) and edema factor (EF)), both of which can share a common B-component known as protective antigen (PA).

[115] *Yersinia pestis* (plague): Plague antigens include F1 capsular antigen (Infect Immun. 2003 Jan; 71(1)): 374-383, LPS (Infect Immun. 1999 Oct; 67(10): 5395), *Yersinia pestis* V antigen (Infect Immun. 1997 Nov; 65(11): 4476-4482).

[116] *Mycobacterium tuberculosis*: Tuberculosis antigens include lipoproteins, LPS, BCG antigens, a fusion protein of antigen 85B (Ag85B) and/or ESAT-6 optionally formulated in cationic lipid vesicles (Infect Immun. 2004 October; 72(10): 6148), *Mycobacterium tuberculosis* (Mtb) isocitrate dehydrogenase associated antigens (Proc Natl Acad Sci U S A. 2004 Aug 24; 101(34): 12652), and/or MPT51 antigens (Infect Immun. 2004 July; 72(7): 3829).

[117] *Rickettsia*: Antigens include outer membrane proteins, including the outer membrane protein A and/or B (OmpB) (Biochim Biophys Acta. 2004 Nov 1;1702(2):145), LPS, and surface protein antigen (SPA) (J Autoimmun. 1989 Jun;2 Suppl:81).

[118] *Listeria monocytogenes*. Bacterial antigens may be derived from Listeria monocytogenes.

[119] *Chlamydia pneumoniae*: Antigens include those identified in WO 02/02606.

[120] *Vibrio cholerae*: Antigens include proteinase antigens, LPS, particularly lipopolysaccharides of *Vibrio cholerae* II, O1 Inaba O-specific polysaccharides, V. cholera O139, antigens of IEM108 vaccine (Infect Immun. 2003 Oct;71(10):5498-504), and/or Zonula occludens toxin (Zot).

[121] *Salmonella typhi* (typhoid fever): Antigens include capsular polysaccharides preferably conjugates (Vi, *i.e.* vax-TyVi).

[122] *Borrelia burgdorferi* (Lyme disease): Antigens include lipoproteins (such as OspA, OspB, Osp C and Osp D), other surface proteins such as OspE-related proteins (Erps), decorin-binding proteins (such as DbpA), and antigenically variable VI proteins. , such as antigens associated with P39 and P13 (an integral membrane protein, Infect Immun. 2001 May; 69(5): 3323-3334), VlsE Antigenic Variation Protein (J Clin Microbiol. 1999 Dec; 37(12): 3997).

[123] *Porphyromonas gingivalis*: Antigens include *P. gingivalis* outer membrane protein (OMP).

[124] *Klebsiella*: Antigens include an OMP, including OMP A, or a polysaccharide optionally conjugated to tetanus toxoid.

[125] Further bacterial antigens of the invention may be capsular antigens, polysaccharide antigens or protein antigens of any of the above. Further bacterial antigens may also include an outer membrane vesicle (OMV) preparation. Additionally, antigens include live, attenuated, and/or purified versions of any of the aforementioned bacteria. The

antigens of the present invention may be derived from gram-negative or gram-positive bacteria. The antigens of the present invention may be derived from aerobic or anaerobic bacteria.

[126] Additionally, any of the above bacterial-derived saccharides (polysaccharides, LPS, LOS or oligosaccharides) can be conjugated to another agent or antigen, such as a carrier protein (for example CRM197). Such conjugation may be direct conjugation effected by reductive amination of carbonyl moieties on the saccharide to amino groups on the protein, as provided in US Patent No. 5,360,897 and Can J Biochem Cell Biol. 1984 May;62(5):270-5. Alternatively, the saccharides can be conjugated through a linker, such as, with succinamide or other linkages provided in Bioconjugate Techniques, 1996 and CRC, Chemistry of Protein Conjugation and Cross-Linking, 1993.

B. Viral Antigens

[127] Viral antigens suitable for use in the invention include inactivated (or killed) virus, attenuated virus, split virus formulations, purified subunit formulations, viral proteins which may be isolated, purified or derived from a virus, and Virus Like Particles (VLPs). Viral antigens may be derived from viruses propagated on cell culture or other substrate. Alternatively, viral antigens may be expressed recombinantly. Viral antigens preferably include epitopes which are exposed on the surface of the virus during at least one stage of its life cycle. Viral antigens are preferably conserved across multiple serotypes or isolates. Viral antigens include antigens derived from one or more of the viruses set forth below as well as the specific antigens examples identified below.

[128] *Orthomyxovirus*: Viral antigens may be derived from an *Orthomyxovirus*, such as Influenza A, B and C. *Orthomyxovirus* antigens may be selected from one or more of the viral proteins, including hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix protein (M1), membrane protein (M2), one or more of the transcriptase components (PB1, PB2 and PA). Preferred antigens include HA and NA.

[129] Influenza antigens may be derived from interpandemic (annual) flu strains. Alternatively influenza antigens may be derived from strains with the potential to cause pandemic a

pandemic outbreak (*i.e.*, influenza strains with new haemagglutinin compared to the haemagglutinin in currently circulating strains, or influenza strains which are pathogenic in avian subjects and have the potential to be transmitted horizontally in the human population, or influenza strains which are pathogenic to humans).

[130] Paramyxoviridae viruses: Viral antigens may be derived from Paramyxoviridae viruses, such as Pneumoviruses (RSV), Paramyxoviruses (PIV) and Morbilliviruses (Measles).

[131] Pneumovirus: Viral antigens may be derived from a Pneumovirus, such as Respiratory syncytial virus (RSV), Bovine respiratory syncytial virus, Pneumonia virus of mice, and Turkey rhinotracheitis virus. Preferably, the Pneumovirus is RSV. Pneumovirus antigens may be selected from one or more of the following proteins, including surface proteins Fusion (F), Glycoprotein (G) and Small Hydrophobic protein (SH), matrix proteins M and M2, nucleocapsid proteins N, P and L and nonstructural proteins NS1 and NS2. Preferred Pneumovirus antigens include F, G and M. See *e.g.*, J Gen Virol. 2004 Nov; 85(Pt 11):3229). Pneumovirus antigens may also be formulated in or derived from chimeric viruses. For example, chimeric RSV/PIV viruses may comprise components of both RSV and PIV.

[132] Paramyxovirus: Viral antigens may be derived from a Paramyxovirus, such as Parainfluenza virus types 1 – 4 (PIV), Mumps, Sendai viruses, Simian virus 5, Bovine parainfluenza virus and Newcastle disease virus. Preferably, the Paramyxovirus is PIV or Mumps. Paramyxovirus antigens may be selected from one or more of the following proteins: Hemagglutinin –Neuraminidase (HN), Fusion proteins F1 and F2, Nucleoprotein (NP), Phosphoprotein (P), Large protein (L), and Matrix protein (M). Preferred Paramyxovirus proteins include HN, F1 and F2. Paramyxovirus antigens may also be formulated in or derived from chimeric viruses. For example, chimeric RSV/PIV viruses may comprise components of both RSV and PIV. Commercially available mumps vaccines include live attenuated mumps virus, in either a monovalent form or in combination with measles and rubella vaccines (MMR).

[133] Morbillivirus: Viral antigens may be derived from a Morbillivirus, such as Measles. Morbillivirus antigens may be selected from one or more of the following proteins: hemagglutinin (H), Glycoprotein (G), Fusion factor (F), Large protein (L), Nucleoprotein (NP), Polymerase phosphoprotein (P), and Matrix (M). Commercially available measles vaccines include live attenuated measles virus, typically in combination with mumps and rubella (MMR).

[134] Picornavirus: Viral antigens may be derived from Picornaviruses, such as Enteroviruses, Rhinoviruses, Heparnavirus, Cardioviruses and Aphthoviruses. Antigens derived from Enteroviruses, such as Poliovirus are preferred.

[135] Enterovirus: Viral antigens may be derived from an Enterovirus, such as Poliovirus types 1, 2 or 3, Coxsackie A virus types 1 to 22 and 24, Coxsackie B virus types 1 to 6, Echovirus (ECHO) virus) types 1 to 9, 11 to 27 and 29 to 34 and Enterovirus 68 to 71. Preferably, the Enterovirus is poliovirus. Enterovirus antigens are preferably selected from one or more of the following Capsid proteins VP1, VP2, VP3 and VP4. Commercially available polio vaccines include Inactivated Polio Vaccine (IPV) and Oral poliovirus vaccine (OPV).

[136] Heparnavirus: Viral antigens may be derived from an Heparnavirus, such as Hepatitis A virus (HAV). Commercially available HAV vaccines include inactivated HAV vaccine.

[137] Togavirus: Viral antigens may be derived from a Togavirus, such as a Rubivirus, an Alphavirus, or an Arterivirus. Antigens derived from Rubivirus, such as Rubella virus, are preferred. Togavirus antigens may be selected from E1, E2, E3, C, NSP-1, NSPO-2, NSP-3 or NSP-4. Togavirus antigens are preferably selected from E1, E2 or E3. Commercially available Rubella vaccines include a live cold-adapted virus, typically in combination with mumps and measles vaccines (MMR).

[138] Flavivirus: Viral antigens may be derived from a Flavivirus, such as Tick-borne encephalitis (TBE), Dengue (types 1, 2, 3 or 4), Yellow Fever, Japanese encephalitis, West Nile encephalitis, St. Louis encephalitis, Russian spring-summer encephalitis, Powassan encephalitis. Flavivirus antigens may be selected from PrM, M, C, E, NS-1,

NS-2a, NS2b, NS3, NS4a, NS4b, and NS5. Flavivirus antigens are preferably selected from PrM, M and E. Commercially available TBE vaccine include inactivated virus vaccines.

[139] Pestivirus: Viral antigens may be derived from a Pestivirus, such as Bovine viral diarrhea (BVDV), Classical swine fever (CSFV) or Border disease (BDV).

[140] Hepadnavirus: Viral antigens may be derived from a Hepadnavirus, such as Hepatitis B virus. Hepadnavirus antigens may be selected from surface antigens (L, M and S), core antigens (HBc, HBe). Commercially available HBV vaccines include subunit vaccines comprising the surface antigen S protein.

[141] Hepatitis C virus: Viral antigens may be derived from a Hepatitis C virus (HCV). HCV antigens may be selected from one or more of E1, E2, E1/E2, NS345 polyprotein, NS 345-core polyprotein, core, and/or peptides from the nonstructural regions (Houghton *et al.*, Hepatology (1991) 14:381).

[142] Rhabdovirus: Viral antigens may be derived from a Rhabdovirus, such as a Lyssavirus (Rabies virus) and Vesiculovirus (VSV). Rhabdovirus antigens may be selected from glycoprotein (G), nucleoprotein (N), large protein (L), nonstructural proteins (NS). Commercially available Rabies virus vaccine comprise killed virus grown on human diploid cells or fetal rhesus lung cells.

[143] Caliciviridae; Viral antigens may be derived from Calciviridae, such as Norwalk virus, and Norwalk-like Viruses, such as Hawaii Virus and Snow Mountain Virus.

[144] Coronavirus: Viral antigens may be derived from a Coronavirus, SARS, Human respiratory coronavirus, Avian infectious bronchitis (IBV), Mouse hepatitis virus (MHV), and Porcine transmissible gastroenteritis virus (TGEV). Coronavirus antigens may be selected from spike (S), envelope (E), matrix (M), nucleocapsid (N), and Hemagglutinin-esterase glycoprotein (HE). Preferably, the Coronavirus antigen is derived from a SARS virus. SARS viral antigens are described in WO 04/92360;

[145] Retrovirus: Viral antigens may be derived from a Retrovirus, such as an Oncovirus, a Lentivirus or a Spumavirus. Oncovirus antigens may be derived from HTLV-1, HTLV-2 or HTLV-5. Lentivirus antigens may be derived from HIV-1 or HIV-2. Retrovirus antigens may be selected from gag, pol, env, tax, tat, rex, rev, nef, vif, vpu, and vpr. HIV antigens may be selected from gag (p24gag and p55gag), env (gp160 and gp41), pol, tat, nef, rev vpu, miniproteins, (preferably p55 gag and gp140v delete). HIV antigens may be derived from one or more of the following strains: HIVIIIb, HIVSF2, HIVLAV, HIVLAI, HIVMN, HIV-1CM235, HIV-1US4.

[146] Reovirus: Viral antigens may be derived from a Reovirus, such as an Orthoreovirus, a Rotavirus, an Orbivirus, or a Coltivirus. Reovirus antigens may be selected from structural proteins λ1, λ2, λ3, μ1, μ2, σ1, σ2, or σ3, or nonstructural proteins σNS, μNS, or σ1s. Preferred Reovirus antigens may be derived from a Rotavirus. Rotavirus antigens may be selected from VP1, VP2, VP3, VP4 (or the cleaved product VP5 and VP8), NSP 1, VP6, NSP3, NSP2, VP7, NSP4, or NSP5. Preferred Rotavirus antigens include VP4 (or the cleaved product VP5 and VP8), and VP7.

[147] Parvovirus: Viral antigens may be derived from a Parvovirus, such as Parvovirus B19. Parvovirus antigens may be selected from VP-1, VP-2, VP-3, NS-1 and NS-2. Preferably, the Parvovirus antigen is capsid protein VP-2.

[148] Delta hepatitis virus (HDV): Viral antigens may be derived HDV, particularly δ-antigen from HDV (see, *e.g.*, U.S. Patent No. 5,378,814).

[149] Hepatitis E virus (HEV): Viral antigens may be derived from HEV.

[150] Hepatitis G virus (HGV): Viral antigens may be derived from HGV.

[151] Human Herpesvirus: Viral antigens may be derived from a Human Herpesvirus, such as Herpes Simplex Viruses (HSV), Varicella-zoster virus (VZV), Epstein-Barr virus (EBV), Cytomegalovirus (CMV), Human Herpesvirus 6 (HHV6), Human Herpesvirus 7 (HHV7), and Human Herpesvirus 8 (HHV8). Human Herpesvirus antigens may be selected from immediate early proteins (α), early proteins (β), and late proteins (γ). HSV

antigens may be derived from HSV-1 or HSV-2 strains. HSV antigens may be selected from glycoproteins gB, gC, gD and gH, fusion protein (gB), or immune escape proteins (gC, gE, or gI). VZV antigens may be selected from core, nucleocapsid, tegument, or envelope proteins. A live attenuated VZV vaccine is commercially available. EBV antigens may be selected from early antigen (EA) proteins, viral capsid antigen (VCA), and glycoproteins of the membrane antigen (MA). CMV antigens may be selected from capsid proteins, envelope glycoproteins (such as gB and gH), and tegument proteins

[152] Papovaviruses: Antigens may be derived from Papovaviruses, such as Papillomaviruses and Polyomaviruses. Papillomaviruses include HPV serotypes 1, 2, 4, 5, 6, 8, 11, 13, 16, 18, 31, 33, 35, 39, 41, 42, 47, 51, 57, 58, 63 and 65. Preferably, HPV antigens are derived from serotypes 6, 11, 16 or 18. HPV antigens may be selected from capsid proteins (L1) and (L2), or E1 – E7, or fusions thereof. HPV antigens are preferably formulated into virus-like particles (VLPs). Polyomyavirus viruses include BK virus and JK virus. Polyomavirus antigens may be selected from VP1, VP2 or VP3.

[153] Further provided are antigens, compositions, methods, and microbes included in Vaccines, 4th Edition (Plotkin and Orenstein ed. 2004); Medical Microbiology 4th Edition (Murray *et al.* ed. 2002); Virology, 3rd Edition (W.K. Joklik ed. 1988); Fundamental Virology, 2nd Edition (B.N. Fields and D.M. Knipe, eds. 1991), which are contemplated in conjunction with the compositions of the present invention.

C. Fungal Antigens

[154] Fungal antigens for use in the invention may be derived from one or more of the fungi set forth below.

[155] Fungal antigens may be derived from Dermatophytres, including: Epidermophyton floccusum, Microsporum audouini, Microsporum canis, Microsporum distortum, Microsporum equinum, Microsporum gypsum, Microsporum nanum, Trichophyton concentricum, Trichophyton equinum, Trichophyton gallinae, Trichophyton gypseum, Trichophyton megnini, Trichophyton mentagrophytes, Trichophyton quinckeanum, Trichophyton rubrum, Trichophyton schoenleini, Trichophyton tonsurans, Trichophyton

verrucosum, T. verrucosum var. album, var. discoides, var. ochraceum, Trichophyton violaceum, and/or Trichophyton faviforme.

[156] Fungal pathogens may be derived from Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger, Aspergillus nidulans, Aspergillus terreus, Aspergillus sydowi, Aspergillus flavatus, Aspergillus glaucus, Blastoschizomyces capitatus, Candida albicans, Candida enolase, Candida tropicalis, Candida glabrata, Candida krusei, Candida parapsilosis, Candida stellatoidea, Candida kusei, Candida parakwsei, Candida lusitaniae, Candida pseudotropicalis, Candida guilliermondi, Cladosporium carrionii, Coccidioides immitis, Blastomyces dermatidis, Cryptococcus neoformans, Geotrichum clavatum, Histoplasma capsulatum, Klebsiella pneumoniae, Paracoccidioides brasiliensis, Pneumocystis carinii, Pythiumn insidiosum, Pityrosporum ovale, Sacharomyces cerevisae, Saccharomyces boulardii, Saccharomyces pombe, Scedosporium apiosperum, Sporothrix schenckii, Trichosporon beigelii, Toxoplasma gondii, Penicillium marneffei, Malassezia spp., Fonsecaea spp., Wangiella spp., Sporothrix spp., Basidiobolus spp., Conidiobolus spp., Rhizopus spp, Mucor spp, Absidia spp, Mortierella spp, Cunninghamella spp, Saksenaea spp., Alternaria spp, Curvularia spp, Helminthosporium spp, Fusarium spp, Aspergillus spp, Penicillium spp, Monolinia spp, Rhizoctonia spp, Paecilomyces spp, Pithomyces spp, and Cladosporium spp.

[157] Processes for producing a fungal antigens are well known in the art (see US Patent No. 6,333,164). In a preferred method a solubilized fraction extracted and separated from an insoluble fraction obtainable from fungal cells of which cell wall has been substantially removed or at least partially removed, characterized in that the process comprises the steps of: obtaining living fungal cells; obtaining fungal cells of which cell wall has been substantially removed or at least partially removed; bursting the fungal cells of which cell wall has been substantially removed or at least partially removed; obtaining an insoluble fraction; and extracting and separating a solubilized fraction from the insoluble fraction.

D. STD Antigens

[158] The compositions of the invention may include one or more antigens derived from a sexually transmitted disease (STD). Such antigens may provide for prophylactis or therapy for STD's such as chlamydia, genital herpes, hepatits (such as HCV), genital warts, gonorrhoea, syphilis and/or chancroid (See, WO00/15255). Antigens may be derived from one or more viral or bacterial STD's. Viral STD antigens for use in the invention may be derived from, for example, HIV, herpes simplex virus (HSV-1 and HSV-2), human papillomavirus (HPV), and hepatitis (HCV). Bacterial STD antigens for use in the invention may be derived from, for example, Neiserria gonorrhoeae, Chlamydia trachomatis, Treponema pallidum, Haemophilus ducreyi, E. coli, and Streptococcus agalactiae. Examples of specific antigens derived from these pathogens are described above.

E. Respiratory Antigens

[159] The compositions of the invention may include one or more antigens derived from a pathogen which causes respiratory disease. For example, respiratory antigens may be derived from a respiratory virus such as Orthomyxoviruses (influenza), Pneumovirus (RSV), Paramyxovirus (PIV), Morbillivirus (measles), Togavirus (Rubella), VZV, and Coronavirus (SARS). Respiratory antigens may be derived from a bacteria which causes respiratory disease, such as Streptococcus pneumoniae, Pseudomonas aeruginosa, Bordetella pertussis, Mycobacterium tuberculosis, Mycoplasma pneumoniae, Chlamydia pneumoniae, Bacillus anthracis, and Moraxella catarrhalis. Examples of specific antigens derived from these pathogens are described above.

F. Pediatric Vaccine Antigens

[160] The compositions of the invention may include one or more antigens suitable for use in pediatric subjects. Pediatric subjects are typically less than about 3 years old, or less than about 2 years old, or less than about 1 years old. Pediatric antigens may be administered multiple times over the course of 6 months, 1, 2 or 3 years. Pediatric antigens may be derived from a virus which may target pediatric populations and/or a virus from which pediatric populations are susceptible to infection. Pediatric viral antigens include

antigens derived from one or more of Orthomyxovirus (influenza), Pneumovirus (RSV), Paramyxovirus (PIV and Mumps), Morbillivirus (measles), Togavirus (Rubella), Enterovirus (polio), HBV, Coronavirus (SARS), and Varicella-zoster virus (VZV), Epstein Barr virus (EBV). Pediatric bacterial antigens include antigens derived from one or more of Streptococcus pneumoniae, Neisseria meningitides, Streptococcus pyogenes (Group A Streptococcus), Moraxella catarrhalis, Bordetella pertussis, Staphylococcus aureus, Clostridium tetani (Tetanus), Cornynebacterium diphtheriae (Diphtheria), Haemophilus influenzae B (Hib), Pseudomonas aeruginosa, Streptococcus agalactiae (Group B Streptococcus), and E. coli. Examples of specific antigens derived from these pathogens are described above.

G. Antigens suitable for use in Elderly or Immunocompromised Individuals

[161] The compositions of the invention may include one or more antigens suitable for use in elderly or immunocompromised individuals. Such individuals may need to be vaccinated more frequently, with higher doses or with adjuvanted formulations to improve their immune response to the targeted antigens. Antigens which may be targeted for use in Elderly or Immunocompromised individuals include antigens derived from one or more of the following pathogens: Neisseria meningitides, Streptococcus pneumoniae, Streptococcus pyogenes (Group A Streptococcus), Moraxella catarrhalis, Bordetella pertussis, Staphylococcus aureus, Staphylococcus epidermis, Clostridium tetani (Tetanus), Cornynebacterium diphtheriae (Diphtheria), Haemophilus influenzae B (Hib), Pseudomonas aeruginosa, Legionella pneumophila, Streptococcus agalactiae (Group B Streptococcus), Enterococcus faecalis, Helicobacter pylori, Clamydia pneumoniae, Orthomyxovirus (influenza), Pneumovirus (RSV), Paramyxovirus (PIV and Mumps), Morbillivirus (measles), Togavirus (Rubella), Enterovirus (polio), HBV, Coronavirus (SARS), Varicella-zoster virus (VZV), Epstein Barr virus (EBV), Cytomegalovirus (CMV). Examples of specific antigens derived from these pathogens are described above.

H. Antigens suitable for use in Adolescent Vaccines

[162] The compositions of the invention may include one or more antigens suitable for use in adolescent subjects. Adolescents may be in need of a boost of a previously administered pediatric antigen. Pediatric antigens which may be suitable for use in adolescents are described above. In addition, adolescents may be targeted to receive antigens derived from an STD pathogen in order to ensure protective or therapeutic immunity before the beginning of sexual activity. STD antigens which may be suitable for use in adolescents are described above.

I. Antigen Formulations

[163] In other aspects of the invention, methods of producing microparticles having adsorbed antigens are provided. The methods comprise: (a) providing an emulsion by dispersing a mixture comprising (i) water, (ii) a detergent, (iii) an organic solvent, and (iv) a biodegradable polymer selected from the group consisting of a poly(α-hydroxy acid), a polyhydroxy butyric acid, a polycaprolactone, a polyorthoester, a polyanhydride, and a polycyanoacrylate. The polymer is typically present in the mixture at a concentration of about 1% to about 30% relative to the organic solvent, while the detergent is typically present in the mixture at a weight-to-weight detergent-to-polymer ratio of from about 0.00001:1 to about 0.1:1 (more typically about 0.0001:1 to about 0.1:1, about 0.001:1 to about 0.1:1, or about 0.005:1 to about 0.1:1); (b) removing the organic solvent from the emulsion; and (c) adsorbing an antigen on the surface of the microparticles. In certain embodiments, the biodegradable polymer is present at a concentration of about 3% to about 10% relative to the organic solvent.

[164] Microparticles for use herein will be formed from materials that are sterilizable, non-toxic and biodegradable. Such materials include, without limitation, poly(α-hydroxy acid), polyhydroxybutyric acid, polycaprolactone, polyorthoester, polyanhydride, PACA, and polycyanoacrylate. Preferably, microparticles for use with the present invention are derived from a poly(α-hydroxy acid), in particular, from a poly(lactide) ("PLA") or a copolymer of D,L-lactide and glycolide or glycolic acid, such as a poly(D,L-lactide-co-

glycolide) ("PLG" or "PLGA"), or a copolymer of D,L-lactide and caprolactone. The microparticles may be derived from any of various polymeric starting materials which have a variety of molecular weights and, in the case of the copolymers such as PLG, a variety of lactide:glycolide ratios, the selection of which will be largely a matter of choice, depending in part on the coadministered macromolecule. These parameters are discussed more fully below.

[165] Further antigens may also include an outer membrane vesicle (OMV) preparation.

[166] Additional formulation methods and antigens (especially tumor antigens) are provided in U.S. Patent Serial No. 09/581,772.

J. Antigen References

[167] The following references include antigens useful in conjunction with the compositions of the present invention:

1 International patent application WO99/24578
2 International patent application WO99/36544.
3 International patent application WO99/57280.
4 International patent application WO00/22430.
5 Tettelin *et al.* (2000) Science 287:1809-1815.
6 International patent application WO96/29412.
7 Pizza *et al.* (2000) Science 287:1816-1820.
8 PCT WO 01/52885.
9 Bjune *et al.* (1991) Lancet 338(8775).
10 Fuskasawa *et al.* (1999) Vaccine 17:2951-2958.
11 Rosenqist *et al.* (1998) Dev. Biol. Strand 92:323-333.
12 Constantino *et al.* (1992) Vaccine 10:691-698.
13 Constantino *et al.* (1999) Vaccine 17:1251-1263.
14 Watson (2000) Pediatr Infect Dis J 19:331-332.
15 Rubin (20000) Pediatr Clin North Am 47:269-285,v.
16 Jedrzejas (2001) Microbiol Mol Biol Rev 65:187-207.
17 International patent application filed on 3rd July 2001 claiming priority from GB-0016363.4;WO 02/02606; PCT IB/01/00166.
18 Kalman *et al.* (1999) Nature Genetics 21:385-389.
19 Read *et al.* (2000) Nucleic Acids Res 28:1397-406.
20 Shirai *et al.* (2000) J. Infect. Dis 181(Suppl 3):S524-S527.
21 International patent application WO99/27105.
22 International patent application WO00/27994.

23 International patent application WO00/37494.
24 International patent application WO99/28475.
25 Bell (2000) Pediatr Infect Dis J 19:1187-1188.
26 Iwarson (1995) APMIS 103:321-326.
27 Gerlich *et al.* (1990) Vaccine 8 Suppl:S63-68 & 79-80.
28 Hsu *et al.* (1999) Clin Liver Dis 3:901-915.
29 Gastofsson *et al.* (1996) N. Engl. J. Med. 334-:349-355.
30 Rappuoli *et al.* (1991) TIBTECH 9:232-238.
31 Vaccines (1988) eds. Plotkin & Mortimer. ISBN 0-7216-1946-0.
32 Del Guidice *et al.* (1998) Molecular Aspects of Medicine 19:1-70.
33 International patent application WO93/018150.
34 International patent application WO99/53310.
35 International patent application WO98/04702.
36 Ross *et al.* (2001) Vaccine 19:135-142.
37 Sutter *et al.* (2000) Pediatr Clin North Am 47:287-308.
38 Zimmerman & Spann (1999) Am Fan Physician 59:113-118, 125-126.
39 Dreensen (1997) Vaccine 15 Suppl"S2-6.
40 MMWR Morb Mortal Wkly rep 1998 Jan 16:47(1):12, 9.
41 McMichael (2000) Vaccine19 Suppl 1:S101-107.
42 Schuchat (1999) Lancer 353(9146):51-6.
43 GB patent applications 0026333.5, 0028727.6 & 0105640.7.
44 Dale (1999) Infect Disclin North Am 13:227-43, viii.
45 Ferretti *et al.* (2001) PNAS USA 98: 4658-4663.
46 Kuroda *et al.* (2001) Lancet 357(9264):1225-1240; see also pages 1218-1219.
47 Ramsay *et al.* (2001) Lancet 357(9251):195-196.
48 Lindberg (1999) Vaccine 17 Suppl 2:S28-36.
49 Buttery & Moxon (2000) J R Coil Physicians Long 34:163-168.
50 Ahmad & Chapnick (1999) Infect Dis Clin North Am 13:113-133, vii.
51 Goldblatt (1998) J. Med. Microbiol. 47:663-567.
52 European patent 0 477 508.
53 U.S. Patent No. 5,306,492.
54 International patent application WO98/42721.
55 Conjugate Vaccines (eds. Cruse *et al.*) ISBN 3805549326, particularly vol. 10:48-114.
56 Hermanson (1996) Bioconjugate Techniques ISBN: 012323368 & 012342335X.
57 European patent application 0372501.
58 European patent application 0378881.
59 European patent application 0427347.
60 International patent application WO93/17712.
61 International patent application WO98/58668.
62 European patent application 0471177.
63 International patent application WO00/56360.
64 International patent application WO00/67161.

[168] The contents of all of the above cited patents, patent applications and journal articles are incorporated by reference as if set forth fully herein.

[169] Where a saccharide or carbohydrate antigen is used, it is preferably conjugated to a carrier protein in order to enhance immunogenicity. See Ramsay *et al.* (2001) Lancet 357(9251):195-196; Lindberg (1999) Vaccine 17 Suppl 2:S28-36; Buttery & Moxon (2000) J R Coll Physicians Lond 34:163-168; Ahmad & Chapnick (1999) Infect Dis Clin North Am 13:113-133, vii; Goldblatt (1998) J. Med. Microbiol. 47:563-567; European patent 0 477 508; US Patent No. 5,306,492; WO98/42721; Conjugate Vaccines (eds. Cruse *et al.*) ISBN 3805549326, particularly vol. 10:48-114; Hermanson (1996) Bioconjugate Techniques ISBN: 0123423368 or 012342335X. Preferred carrier proteins are bacterial toxins or toxoids, such as diphtheria or tetanus toxoids. The CRM197 diphtheria toxoid is particularly preferred.

[170] Other carrier polypeptides include the *N. meningitidis* outer membrane protein (EP-A-0372501), synthetic peptides (EP-A-0378881 and EP-A 0427347), heat shock proteins (WO 93/17712 and WO 94/03208), pertussis proteins (WO 98/58668 and EP A 0471177), protein D from H. influenzae (WO 00/56360), cytokines (WO 91/01146), lymphokines, hormones, growth factors, toxin A or B from C. difficile (WO 00/61761), iron-uptake proteins (WO 01/72337), etc. Where a mixture comprises capsular saccharide from both serigraphs A and C, it may be preferred that the ratio (w/w) of MenA saccharide:MenC saccharide is greater than 1 (*e.g.*, 2:1, 3:1, 4:1, 5:1, 10:1 or higher). Different saccharides can be conjugated to the same or different type of carrier protein. Any suitable conjugation reaction can be used, with any suitable linker where necessary.

[171] Toxic protein antigens may be detoxified where necessary *e.g.*, detoxification of pertussis toxin by chemical and/or genetic means.

Pharmaceutically acceptable carriers

[172] Compositions of the invention will typically, in addition to the components mentioned above, comprise one or more "pharmaceutically acceptable carriers." These include any carrier which does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers typically are large, slowly metabolized

macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and lipid aggregates (such as oil droplets or liposomes). Such carriers are well known to those of ordinary skill in the art. A composition may also contain a diluent, such as water, saline, glycerol, etc. Additionally, an auxiliary substance, such as a wetting or emulsifying agent, pH buffering substance, and the like, may be present. A thorough discussion of pharmaceutically acceptable components is available in Gennaro (2000) Remington: The Science and Practice of Pharmacy. 20th ed., ISBN: 0683306472.

Immunoregulatory Agents

Adjuvants

[173] Vaccines of the invention may be administered in conjunction with other immunoregulatory agents. In particular, compositions will usually include an adjuvant. Adjuvants for use with the invention include, but are not limited to, one or more of the following set forth below:

A. Mineral Containing Compositions

[174] Mineral containing compositions suitable for use as adjuvants in the invention include mineral salts, such as aluminum salts and calcium salts. The invention includes mineral salts such as hydroxides (*e.g.* oxyhydroxides), phosphates (*e.g.* hydroxyphosphates, orthophosphates), sulfates, etc. (*e.g.* see chapters 8 & 9 of Vaccine Design... (1995) eds. Powell & Newman. ISBN: 030644867X. Plenum.), or mixtures of different mineral compounds (*e.g.* a mixture of a phosphate and a hydroxide adjuvant, optionally with an excess of the phosphate), with the compounds taking any suitable form (*e.g.* gel, crystalline, amorphous, etc.), and with adsorption to the salt(s) being preferred. The mineral containing compositions may also be formulated as a particle of metal salt (WO00/23105).

[175] Aluminum salts may be included in vaccines of the invention such that the dose of Al^{3+} is between 0.2 and 1.0 mg per dose.

[176] In one embodiment the aluminum based adjuvant for use in the present invention is alum (aluminum potassium sulfate ($AlK(SO_4)_2$)), or an alum derivative, such as that formed in-situ by mixing an antigen in phosphate buffer with alum, followed by titration and precipitation with a base such as ammonium hydroxide or sodium hydroxide.

[177] Another aluminum-based adjuvant for use in vaccine formulations of the present invention is aluminum hydroxide adjuvant ($Al(OH)_3$) or crystalline aluminum oxyhydroxide (AlOOH), which is an excellent adsorbant, having a surface area of approximately $500m^2/g$. Alternatively, aluminum phosphate adjuvant ($AlPO_4$) or aluminum hydroxyphosphate, which contains phosphate groups in place of some or all of the hydroxyl groups of aluminum hydroxide adjuvant is provided. Preferred aluminum phosphate adjuvants provided herein are amorphous and soluble in acidic, basic and neutral media.

[178] In another embodiment the adjuvant of the invention comprises both aluminum phosphate and aluminum hydroxide. In a more particular embodiment thereof, the adjuvant has a greater amount of aluminum phosphate than aluminum hydroxide, such as a ratio of 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1 or greater than 9:1, by weight aluminum phosphate to aluminum hydroxide. More particular still, aluminum salts in the vaccine are present at 0.4 to 1.0 mg per vaccine dose, or 0.4 to 0.8 mg per vaccine dose, or 0.5 to 0.7 mg per vaccine dose, or about 0.6 mg per vaccine dose.

[179] Generally, the preferred aluminum-based adjuvant(s), or ratio of multiple aluminum-based adjuvants, such as aluminum phosphate to aluminum hydroxide is selected by optimization of electrostatic attraction between molecules such that the antigen carries an opposite charge as the adjuvant at the desired pH. For example, aluminum phosphate adjuvant (isoelectric point = 4) adsorbs lysozyme, but not albumin at pH 7.4. Should albumin be the target, aluminum hydroxide adjuvant would be selected (iep 11.4). Alternatively, pretreatment of aluminum hydroxide with phosphate lowers its isoelectric point, making it a preferred adjuvant for more basic antigens.

B. Oil-Emulsions

[180] Oil-emulsion compositions suitable for use as adjuvants in the invention include squalene-water emulsions, such as MF59 (5% Squalene, 0.5% TWEEN™ 80, and 0.5% Span 85, formulated into submicron particles using a microfluidizer). See WO90/14837. See also, Podda, Vaccine (2001) 19: 2673-2680; Frey *et al.*, Vaccine (2003) 21:4234-4237. MF59 is used as the adjuvant in the FLUAD™ influenza virus trivalent subunit vaccine.

[181] Particularly preferred adjuvants for use in the compositions are submicron oil-in-water emulsions. Preferred submicron oil-in-water emulsions for use herein are squalene/water emulsions optionally containing varying amounts of MTP-PE, such as a submicron oil-in-water emulsion containing 4-5% w/v squalene, 0.25-1.0% w/v TWEEN™ 80□ (polyoxyelthylenesorbitan monooleate), and/or 0.25-1.0% SPAN 85™ (sorbitan trioleate), and, optionally, N-acetylmuramyl-L-alanyl-D-isogluatminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-huydroxyphosphophoryloxy)-ethylamine (MTP-PE), for example, the submicron oil-in-water emulsion known as "MF59" (International Publication No. WO90/14837; US Patent Nos. 6,299,884 and 6,451,325, and Ott *et al.*, in Vaccine Design: The Subunit and Adjuvant Approach (Powell, M.F. and Newman, M.J. eds.) Plenum Press, New York, 1995, pp. 277-296). MF59 contains 4-5% w/v Squalene (*e.g.* 4.3%), 0.25-0.5% w/v TWEEN™ 80, and 0.5% w/v SPAN 85™ and optionally contains various amounts of MTP-PE, formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA). For example, MTP-PE may be present in an amount of about 0-500 μg/dose, more preferably 0-250 μg/dose and most preferably, 0-100 μg/dose. As used herein, the term "MF59-0" refers to the above submicron oil-in-water emulsion lacking MTP-PE, while the term MF59-MTP denotes a formulation that contains MTP-PE. For instance, "MF59-100" contains 100 μg MTP-PE per dose, and so on. MF69, another submicron oil-in-water emulsion for use herein, contains 4.3% w/v squalene, 0.25% w/v TWEEN™ 80, and 0.75% w/v SPAN 85™ and optionally MTP-PE. Yet another submicron oil-in-water emulsion is MF75, also known as SAF, containing 10% squalene, 0.4% TWEEN™ 80,

5% pluronic-blocked polymer L121, and thr-MDP, also microfluidized into a submicron emulsion. MF75-MTP denotes an MF75 formulation that includes MTP, such as from 100-400 μg MTP-PE per dose.

[182] Submicron oil-in-water emulsions, methods of making the same and immunostimulating agents, such as muramyl peptides, for use in the compositions, are described in detail in WO90/14837 and U.S. Patents 6,299,884 and 6,45 1,325.

[183] Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) may also be used as adjuvants in the invention.

C. Saponin Formulations

[184] Saponin formulations, may also be used as adjuvants in the invention. Saponins are a heterologous group of sterol glycosides and triterpenoid glycosides that are found in the bark, leaves, stems, roots and even flowers of a wide range of plant species. Saponins isolated from the bark of the Quillaia saponaria Molina tree have been widely studied as adjuvants. Saponins can also be commercially obtained from Smilax ornata (sarsaprilla), Gypsophilla paniculata (brides veil), and Saponaria officianalis (soap root). Saponin adjuvant formulations include purified formulations, such as QS21, as well as lipid formulations, such as ISCOMs.

[185] Saponin compositions have been purified using High Performance Thin Layer Chromatography (HP-TLC) and Reversed Phase High Performance Liquid Chromatography (RP-HPLC). Specific purified fractions using these techniques have been identified, including QS7, QS17, QS18, QS21, QH-A, QH-B and QH-C. Preferably, the saponin is QS21. A method of production of QS21 is disclosed in U.S. Patent 5,057,540. Saponin formulations may also comprise a sterol, such as cholesterol (see WO96/33739).

[186] Combinations of saponins and cholesterols can be used to form unique particles called Immunostimulating Complexes (ISCOMs). ISCOMs typically also include a phospholipid such as phosphatidylethanolamine or phosphatidylcholine. Any known

saponin can be used in ISCOMs. Preferably, the ISCOM includes one or more of Quil A, QHA and QHC. ISCOMs are further described in EP0109942, WO96/11711 and WO96/33739. Optionally, the ISCOMS may be devoid of (an) additional detergent(s). See WO00/07621.

[187] A review of the development of saponin based adjuvants can be found in Barr, *et al.*, Advanced Drug Delivery Reviews (1998) 32:247-271. *See also* Sjolander, *et al.*, Advanced Drug Delivery Reviews (1998) 32:321-338.

D. Virosomes and Virus Like Particles (VLPs)

[188] Virosomes and Virus Like Particles (VLPs) can also be used as adjuvants in the invention. These structures generally contain one or more proteins from a virus optionally combined or formulated with a phospholipid. They are generally non-pathogenic, non-replicating and generally do not contain any of the native viral genome. The viral proteins may be recombinantly produced or isolated from whole viruses. These viral proteins suitable for use in virosomes or VLPs include proteins derived from influenza virus (such as HA or NA), Hepatitis B virus (such as core or capsid proteins), Hepatitis E virus, measles virus, Sindbis virus, Rotavirus, Foot-and-Mouth Disease virus, Retrovirus, Norwalk virus, human Papilloma virus, HIV, RNA-phages, Qß-phage (such as coat proteins), GA-phage, fr-phage, AP205 phage, and Ty (such as retrotransposon Ty protein p1). VLPs are discussed further in WO03/024480, WO03/024481, and Niikura *et al.*, Virology (2002) 293:273-280; Lenz *et al.*, Journal of Immunology (2001) 5246-5355; Pinto, *et al.*, Journal of Infectious Diseases (2003) 188:327-338; and Gerber *et al.*, Journal of Virology (2001) 75(10):4752-4760. Virosomes are discussed further in, for example, Gluck *et al.*, Vaccine (2002) 20:B10 –B16. Immunopotentiating reconstituted influenza virosomes (IRIV) are used as the subunit antigen delivery system in the intranasal trivalent INFLEXAL™ product {Mischler & Metcalfe (2002) Vaccine 20 Suppl 5:B17-23} and the INFLUVAC PLUS™ product.

E. Bacterial or Microbial Derivatives

[189] Adjuvants suitable for use in the invention include bacterial or microbial derivatives such as:

(1) Non-toxic derivatives of enterobacterial lipopolysaccharide (LPS)

[190] Such derivatives include Monophosphoryl lipid A (MPL) and 3-O-deacylated MPL (3dMPL). 3dMPL is a mixture of 3 De-O-acylated monophosphoryl lipid A with 4, 5 or 6 acylated chains. A preferred "small particle" form of 3 De-O-acylated monophosphoryl lipid A is disclosed in EP 0 689 454. Such "small particles" of 3dMPL are small enough to be sterile filtered through a 0.22 micron membrane (see EP 0 689 454). Other non-toxic LPS derivatives include monophosphoryl lipid A mimics, such as aminoalkyl glucosaminide phosphate derivatives *e.g.* RC 529. See Johnson *et al.* (1999) Bioorg Med Chem Lett 9:2273-2278.

(2) Lipid A Derivatives

[191] Lipid A derivatives include derivatives of lipid A from Escherichia coli such as OM-174. OM-174 is described for example in Meraldi *et al.*, Vaccine (2003) 21:2485-2491; and Pajak, *et al.*, Vaccine (2003) 21:836-842.

(3) Immunostimulatory oligonucleotides

[192] Immunostimulatory oligonucleotides suitable for use as adjuvants in the invention include nucleotide sequences containing a CpG motif (a sequence containing an unmethylated cytosine followed by guanosine and linked by a phosphate bond). Bacterial double stranded RNA or oligonucleotides containing palindromic or poly(dG) sequences have also been shown to be immunostimulatory.

[193] The CpG's can include nucleotide modifications/analogs such as phosphorothioate modifications and can be double-stranded or single-stranded. Optionally, the guanosine may be replaced with an analog such as 2'-deoxy-7-deazaguanosine. See Kandimalla, *et al.*, Nucleic Acids Research (2003) 31(9): 2393-2400; WO02/26757 and WO99/62923

for examples of possible analog substitutions. The adjuvant effect of CpG oligonucleotides is further discussed in Krieg, Nature Medicine (2003) 9(7): 831-835; McCluskie, *et al.*, FEMS Immunology and Medical Microbiology (2002) 32:179-185; WO98/40100; US Patent No. 6,207,646; US Patent No. 6,239,116 and US Patent No. 6,429,199.

[194] The CpG sequence may be directed to TLR9, such as the motif GTCGTT or TTCGTT. See Kandimalla, *et al.*, Biochemical Society Transactions (2003) 31 (part 3): 654-658. The CpG sequence may be specific for inducing a Th1 immune response, such as a CpG-A ODN, or it may be more specific for inducing a B cell response, such a CpG-B ODN. CpG-A and CpG-B ODNs are discussed in Blackwell, *et al.*, J. Immunol. (2003) 170(8):4061-4068; Krieg, TRENDS in Immunology (2002) 23(2): 64-65 and WO01/95935. Preferably, the CpG is a CpG-A ODN.

[195] Preferably, the CpG oligonucleotide is constructed so that the 5' end is accessible for receptor recognition. Optionally, two CpG oligonucleotide sequences may be attached at their 3' ends to form "immunomers". See, for example, Kandimalla, *et al.*, BBRC (2003) 306:948-953; Kandimalla, *et al.*, Biochemical Society Transactions (2003) 31(part 3):664-658; Bhagat *et al.*, BBRC (2003) 300:853-861 and WO03/035836.

(4) ADP-ribosylating toxins and detoxified derivatives thereof.

[196] Bacterial ADP-ribosylating toxins and detoxified derivatives thereof may be used as adjuvants in the invention. Preferably, the protein is derived from *E. coli* (*i.e.*, *E. coli* heat labile enterotoxin "LT), cholera ("CT"), or pertussis ("PT"). The use of detoxified ADP-ribosylating toxins as mucosal adjuvants is described in WO95/17211 and as parenteral adjuvants in WO98/42375. Preferably, the adjuvant is a detoxified LT mutant such as LT-K63, LT-R72, and LTR192G. The use of ADP-ribosylating toxins and detoxified derivatives thereof, particularly LT-K63 and LT-R72, as adjuvants can be found in the following references: Beignon, *et al.*, Infection and Immunity (2002) 70(6):3012-3019; Pizza, *et al.*, Vaccine (2001) 19:2534-2541; Pizza, *et al.*, Int. J. Med. Microbiol (2000) 290(4-5):455-461; Scharton-Kersten *et al.*, Infection and Immunity (2000) 68(9):5306-

5313; Ryan *et al.*, Infection and Immunity (1999) 67(12):6270-6280; Partidos *et al.*, Immunol. Lett. (1999) 67(3):209-216; Peppoloni *et al.*, Vaccines (2003) 2(2):285-293; and Pine *et al.*, (2002) J. Control Release (2002) 85(1-3):263-270. Numerical reference for amino acid substitutions is preferably based on the alignments of the A and B subunits of ADP-ribosylating toxins set forth in Domenighini *et al.*, Mol. Microbiol (1995) 15(6):1165-1167.

F. Bioadhesives and Mucoadhesives

[197] Bioadhesives and mucoadhesives may also be used as adjuvants in the invention. Suitable bioadhesives include esterified hyaluronic acid microspheres (Singh *et al.* (2001) J. Cont. Rele. 70:267-276) or mucoadhesives such as cross-linked derivatives of polyacrylic acid, polyvinyl alcohol, polyvinyl pyrollidone, polysaccharides and carboxymethylcellulose. Chitosan and derivatives thereof may also be used as adjuvants in the invention. *See* WO99/27960.

G. Microparticles

[198] Microparticles may also be used as adjuvants in the invention. Microparticles (*i.e.* a particle of ~100nm to ~150μm in diameter, more preferably ~200nm to ~30μm in diameter, and most preferably ~500nm to ~10μm in diameter) formed from materials that are biodegradable and non toxic (*e.g.* a poly(α-hydroxy acid), a polyhydroxybutyric acid, a polyorthoester, a polyanhydride, a polycaprolactone, etc.), with poly(lactide co glycolide) are preferred, optionally treated to have a negatively-charged surface (*e.g.* with SDS) or a positively-charged surface (*e.g.* with a cationic detergent, such as CTAB).

H. Liposomes

[199] Examples of liposome formulations suitable for use as adjuvants are described in US Patent No. 6,090,406, US Patent No. 5,916,588, and EP 0 626 169.

I. Polyoxyethylene ether and Polyoxyethylene Ester Formulations

[200] Adjuvants suitable for use in the invention include polyoxyethylene ethers and polyoxyethylene esters. WO99/52549. Such formulations further include polyoxyethylene sorbitan ester surfactants in combination with an octoxynol (WO01/21207) as well as polyoxyethylene alkyl ethers or ester surfactants in combination with at least one additional non-ionic surfactant such as an octoxynol (WO01/21152).

[201] Preferred polyoxyethylene ethers are selected from the following group: polyoxyethylene-9-lauryl ether (laureth 9), polyoxyethylene-9-steoryl ether, polyoxytheylene-8-steoryl ether, polyoxyethylene-4-lauryl ether, polyoxyethylene-35-lauryl ether, and polyoxyethylene-23-lauryl ether.

J. Polyphosphazene (PCPP)

[202] PCPP formulations are described, for example, in Andrianov *et al.*, "Preparation of hydrogel microspheres by coacervation of aqueous polyphophazene solutions", Biomaterials (1998) 19(1-3):109-115 and Payne *et al.*, "Protein Release from Polyphosphazene Matrices", Adv. Drug. Delivery Review (1998) 31(3):185-196.

K. Muramyl peptides

[203] Examples of muramyl peptides suitable for use as adjuvants in the invention include N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-l-alanyl-d-isoglutamine (nor-MDP), and N acetylmuramyl-l-alanyl-d-isoglutaminyl-l-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine MTP-PE).

L. Imidazoquinoline Compounds.

[204] Examples of imidazoquinoline compounds suitable for use adjuvants in the invention include Imiquimod and its analogues, described further in Stanley, Clin Exp Dermatol (2002) 27(7):571-577; Jones, Curr Opin Investig Drugs (2003) 4(2):214-218; and U.S.

Patents 4,689,338, 5,389,640, 5,268,376, 4,929,624, 5,266,575, 5,352,784, 5,494,916, 5,482,936, 5,346,905, 5,395,937, 5,238,944, and 5,525,612.

M. Thiosemicarbazone Compounds.

[205] Examples of thiosemicarbazone compounds, as well as methods of formulating, manufacturing, and screening for compounds all suitable for use as adjuvants in the invention include those described in WO04/60308. The thiosemicarbazones are particularly effective in the stimulation of human peripheral blood mononuclear cells for the production of cytokines, such as TNF- α.

N. Tryptanthrin Compounds.

[206] Examples of tryptanthrin compounds, as well as methods of formulating, manufacturing, and screening for compounds all suitable for use as adjuvants in the invention include those described in WO04/64759. The tryptanthrin compounds are particularly effective in the stimulation of human peripheral blood mononuclear cells for the production of cytokines, such as TNF- α.

[207] The invention may also comprise combinations of aspects of one or more of the adjuvants identified above. For example, the following adjuvant compositions may be used in the invention:

(1) a saponin and an oil-in-water emulsion (WO99/11241);

(2) a saponin (*e.g.*, QS21) + a non-toxic LPS derivative (*e.g.* 3dMPL) (see WO94/00153);

(3) a saponin (*e.g.*, QS21) + a non-toxic LPS derivative (*e.g.* 3dMPL) + a cholesterol;

(4) a saponin (*e.g.*, QS21) + 3dMPL + IL 12 (optionally + a sterol) (WO98/57659);

(5) combinations of 3dMPL with, for example, QS21 and/or oil-in-water emulsions (See European patent applications 0835318, 0735898 and 0761231);

(6) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-block polymer L121, and thr-MDP, either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion.

(7) RIBI™ adjuvant system (RAS), (Ribi Immunochem) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (DETOX™); and

(8) one or more mineral salts (such as an aluminum salt) + a non-toxic derivative of LPS (such as 3dPML).

(9) one or more mineral salts (such as an aluminum salt) + an immunostimulatory oligonucleotide (such as a nucleotide sequence including a CpG motif).

O. Human Immunomodulators

[208] Human immunomodulators suitable for use as adjuvants in the invention include cytokines, such as interleukins (*e.g.* IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, etc.), interferons (*e.g.* interferon-γ), macrophage colony stimulating factor, and tumor necrosis factor.

[209] Aluminum salts and MF59 are preferred adjuvants for use with injectable influenza vaccines. Bacterial toxins and bioadhesives are preferred adjuvants for use with mucosally-delivered vaccines, such as nasal vaccines.

[210] The contents of all of the above cited patents, patent applications and journal articles are incorporated by reference as if set forth fully herein.

Therapeutic methods

[211] The invention provides methods for inducing or increasing an immune response to an SLO antigen using the compositions described above. The immune response is preferably protective and can include antibodies and/or cell-mediated immunity (including systemic and mucosal immunity). Immune responses include booster responses. Compositions comprising antibodies can be used to treat *S. pyogenes* infections.

[212] Teenagers and children, including toddles and infants, can receive a vaccine for prophylactic use; therapeutic vaccines typically are administered to teenagers or adults. A vaccine intended for children may also be administered to adults *e.g.*, to assess safety, dosage, immunogenicity, etc.

[213] Diseases caused by *Streptococcus pyogenes* which can be prevented or treated according to the invention include, but are not limited to, pharyngitis (such as streptococcal sore throat), scarlet fever, impetigo, erysipelas, cellulitis, septicemia, toxic shock syndrome, necrotizing fasciitis, and sequelae such as rheumatic fever and acute glomerulonephritis. The compositions may also be effective against other streptococcal bacteria, *e.g.*, GBS.

Tests to determine the efficacy of the immune response

[214] One way of assessing efficacy of therapeutic treatment involves monitoring GAS infection after administration of the composition of the invention. One way of assessing efficacy of prophylactic treatment involves monitoring immune responses against the SLO antigens in the compositions of the invention after administration of the composition.

[215] Another way of assessing the immunogenicity of the component proteins of the immunogenic compositions of the present invention is to express the proteins recombinantly and to screen patient sera or mucosal secretions by immunoblot. A positive reaction between the protein and the patient serum indicates that the patient has previously mounted an immune response to the protein in question; *i.e.*, the protein is an

immunogen. This method may also be used to identify immunodominant proteins and/or epitopes.

[216] Another way of checking efficacy of therapeutic treatment involves monitoring GAS infection after administration of the compositions of the invention. One way of checking efficacy of prophylactic treatment involves monitoring immune responses both systemically (such as monitoring the level of IgG1 and IgG2a production) and mucosally (such as monitoring the level of IgA production) against the SLO antigens in the compositions of the invention after administration of the composition. Typically, serum specific antibody responses are determined post-immunization but pre-challenge whereas mucosal specific antibody body responses are determined post-immunization and post-challenge.

[217] The vaccine compositions of the present invention can be evaluated in *in vitro* and *in vivo* animal models prior to host, *e.g.*, human, administration. Particularly useful mouse models include those in which intraperitoneal immunization is followed by either intraperitoneal challenge or intranasal challenge.

[218] The efficacy of immunogenic compositions of the invention can also be determined in vivo by challenging animal models of GAS infection, *e.g.*, guinea pigs or mice, with the immunogenic compositions. The immunogenic compositions may or may not be derived from the same serotypes as the challenge serotypes. Preferably the immunogenic compositions are derivable from the same serotypes as the challenge serotypes. More preferably, the immunogenic composition and/or the challenge serotype are derivable from the group of GAS serotypes consisting of M1, M3, M23 and/or combinations thereof.

[219] In vivo efficacy models include but are not limited to: (i) a murine infection model using human GAS serotypes; (ii) a murine disease model which is a murine model using a mouse-adapted GAS strain, such as the M23 strain which is particularly virulent in mice, and (iii) a primate model using human GAS isolates.

[220] The immune response may be one or both of a TH1 immune response and a TH2 response. The immune response may be an improved or an enhanced or an altered immune response. The immune response may be one or both of a systemic and a mucosal immune response. Preferably the immune response is an enhanced system and/or mucosal response.

[221] An enhanced systemic and/or mucosal immunity is reflected in an enhanced TH1 and/or TH2 immune response. Preferably, the enhanced immune response includes an increase in the production of IgG1 and/or IgG2a and/or IgA.

[222] Preferably the mucosal immune response is a TH2 immune response. Preferably, the mucosal immune response includes an increase in the production of IgA.

[223] Activated TH2 cells enhance antibody production and are therefore of value in responding to extracellular infections. Activated TH2 cells may secrete one or more of IL-4, IL-5, IL-6, and IL-10. A TH2 immune response may result in the production of IgG1, IgE, IgA and memory B cells for future protection.

[224] A TH2 immune response may include one or more of an increase in one or more of the cytokines associated with a TH2 immune response (such as IL-4, IL-5, IL-6 and IL-10), or an increase in the production of IgG1, IgE, IgA and memory B cells. Preferably, the enhanced TH2 immune response will include an increase in IgG1 production.

[225] A TH1 immune response may include one or more of an increase in CTLs, an increase in one or more of the cytokines associated with a TH1 immune response (such as IL-2, IFNγ, and TNFβ), an increase in activated macrophages, an increase in NK activity, or an increase in the production of IgG2a. Preferably, the enhanced TH1 immune response will include an increase in IgG2a production.

[226] Immunogenic compositions of the invention, in particular, immunogenic composition comprising one or more SLO antigens of the present invention may be used either alone or in combination with other GAS antigens optionally with an immunoregulatory agent capable of eliciting a Th1 and/or Th2 response.

[227] The invention also comprises an immunogenic composition comprising one or more immunoregulatory agent, such as a mineral salt, such as an aluminium salt and an oligonucleotide containing a CpG motif. Most preferably, the immunogenic composition includes both an aluminium salt and an oligonucleotide containing a CpG motif. Alternatively, the immunogenic composition includes an ADP ribosylating toxin, such as a detoxified ADP ribosylating toxin and an oligonucleotide containing a CpG motif. Preferably, one or more of the immunoregulatory agents include an adjuvant. The adjuvant may be selected from one or more of the group consisting of a TH1 adjuvant and TH2 adjuvant, further discussed below.

[228] The compositions of the invention will preferably elicit both a cell mediated immune response as well as a humoral immune response in order to effectively address a GAS infection. This immune response will preferably induce long lasting (*e.g.*, neutralizing) antibodies and a cell mediated immunity that can quickly respond upon exposure to one or more GAS antigens.

[229] In one particularly preferred embodiment, the immunogenic composition comprises one or more SLO antigen(s) which elicits a neutralizing antibody response and one or more SLO antigen(s) which elicit a cell mediated immune response. In this way, the neutralizing antibody response prevents or inhibits an initial GAS infection while the cell-mediated immune response capable of eliciting an enhanced Th1 cellular response prevents further spreading of the GAS infection.

[230] Compositions of the invention will generally be administered directly to a patient. The compositions of the present invention may be administered, either alone or as part of a composition, via a variety of different routes. Certain routes may be favored for certain compositions, as resulting in the generation of a more effective immune response, preferably a CMI response, or as being less likely to induce side effects, or as being easier for administration.

[231] Delivery methods include parenteral injection (*e.g.*, subcutaneous, intraperitoneal, intravenous, intramuscular, or interstitial injection) and rectal, oral (*e.g.*, tablet, spray),

vaginal, topical, transdermal (*e.g.*, see WO 99/27961), transcutaneous (*e.g.*, see WO02/074244 and WO02/064162), intranasal (*e.g.*, see WO03/028760), ocular, aural, and pulmonary or other mucosal administration.

[232] By way of example, the compositions of the present invention may be administered via a systemic route or a mucosal route or a transdermal route or it may be administered directly into a specific tissue. As used herein, the term "systemic administration" includes but is not limited to any parenteral routes of administration. In particular, parenteral administration includes but is not limited to subcutaneous, intraperitoneal, intravenous, intraarterial, intramuscular, or intrasternal injection, intravenous, intraarterial, or kidney dialytic infusion techniques. Preferably, the systemic, parenteral administration is intramuscular injection. As used herein, the term "mucosal administration" includes but is not limited to oral, intranasal, intravaginal, intrarectal, intratracheal, intestinal and ophthalmic administration.

[233] Dosage treatment can be a single dose schedule or a multiple dose schedule. Multiple doses may be used in a primary immunization schedule and/or in a booster immunization schedule. In a multiple dose schedule the various doses may be given by the same or different routes *e.g.*, a parenteral prime and mucosal boost, a mucosal prime and parenteral boost, etc.

[234] The compositions of the invention may be prepared in various forms. For example, a composition can be prepared as an injectable, either as a liquid solution or a suspension. Solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared (*e.g.*, a lyophilized composition). A composition can be prepared for oral administration, such as a tablet or capsule, as a spray, or as a syrup (optionally flavored). A composition can be prepared for pulmonary administration, *e.g.*, as an inhaler, using a fine powder or a spray. A composition can be prepared as a suppository or pessary. A composition can be prepared for nasal, aural or ocular administration *e.g.*, as drops. A composition can be in kit form, designed such that a combined composition is reconstituted just prior to administration to a patient. Such kits may comprise one or more SLO or other antigens in liquid form and one or more lyophilized antigens.

[235] Immunogenic compositions used as vaccines comprise an immunologically effective amount of SLO or other antigens (or nucleic acid molecules encoding the antigens) or antibodies, as well as any other components, as needed, such as antibiotics. An "immunologically effective amount" is an amount which, when administered to an individual, either in a single dose or as part of a series, increases a measurable immune response or prevents or reduces a clinical symptom.

[236] The immunogenic compositions of the present invention may be administered in combination with an antibiotic treatment regime. In one embodiment, the antibiotic is administered prior to administration of the antigen of the invention or the composition comprising the one or more SLO antigens of the invention.

[237] In another embodiment, the antibiotic is administered subsequent to the administration of the one or more surface-exposed and/or surface-associated SLO antigens of the invention or the composition comprising the one or more surface-exposed and/or surface-associated SLO antigens of the invention. Examples of antibiotics suitable for use in the treatment of a GAS infection include but are not limited to penicillin or a derivative thereof or clindamycin, cephalosporins, glycopeptides (*e.g.*, vancomycin), and cycloserine.

[238] The amount of active agent in a composition varies, however, depending upon the health and physical condition of the individual to be treated, age, the taxonomic group of individual to be treated (*e.g.*, non-human primate, primate, etc.), the capacity of the individual's immune system to synthesize antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. The amount will fall in a relatively broad range which can be determined through routine trials.

Kits

[239] The invention also provides kits comprising one or more containers of compositions of the invention. Compositions can be in liquid form or can be lyophilized, as can individual antigens. Suitable containers for the compositions include, for example, bottles, vials, syringes, and test tubes. Containers can be formed from a variety of

materials, including glass or plastic. A container may have a sterile access port (for example, the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle).

[240] The kit can further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution, or dextrose solution. It can also contain other materials useful to the end-user, including other buffers, diluents, filters, needles, and syringes. The kit can also comprise a second or third container with another active agent, for example an antibiotic.

[241] The kit can also comprise a package insert containing written instructions for methods of inducing immunity against *S. pyogenes* or for treating *S. pyogenes* infections. The package insert can be an unapproved draft package insert or can be a package insert approved by the Food and Drug Administration (FDA) or other regulatory body.

[242] All patents, patent applications, and references cited in this disclosure are expressly incorporated herein by reference. The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples, which are provided for purposes of illustration only and are not intended to limit the scope of the invention.

EXAMPLE 1

[243] The 3D crystal structure of the perfringolysin O monomer from *Clostridium perfringens* has recently been described, using X-ray crystallography, as an elongated molecule comprised of four L-sheet-rich domains, only one of which, the C-terminal domain 4, is contiguous within the primary amino acid sequence (see FIG. 1). GAS25 has homology with this protein, as shown in FIG. 2.

[244] On the basis of the protein sequence homology with *Clostridium perfringens* Perfringolysin O, four domains can be predicted in SLO, which are distributed along the primary sequence as depicted in the scheme shown in FIG. 3.

[245] "Peptide 1" (36-QNTASTETTTTNEQPKPESSELTTEK-61; SEQ ID NO:1), "peptide 2" (155-NINTTPVDISIIDSVTDR-172; SEQ ID NO:4), and "peptide 3" (450-TEYVETTSTEY-460; SEQ ID NO:3) were identified by surfome analysis in SF370-M1 (pep1 and pep2) and in 3650-M6 (pep3) strains. The peptides are underlined in black in FIG. 3.

EXAMPLE 2

Cloning and expression of distinct protein regions

[246] Peptide 1 appears to be located in the 100 amino acid-unstructured amino terminal protein region, while peptide 2 and peptide 3 are almost entirely included in the discontinuous domain 2. Based on this structure prediction, cloning and expression of different protein regions were planned. One protein region included peptide 1 only. Another a protein region included both peptide 2 and peptide 3 ("peptide 2+3"), which required the joining of protein stretches which are not continuous in the primary sequence. The latter fusion was planned in a way which could possibly preserve the structure of the domains that include the two peptides (see FIG. 4). To increase the possibility to achieve this result, isoleucine 165 was replaced with a proline residue, which was expected to favor structural bending, while the naturally existing glycine 445 residue was expected to function as a linker between the two fused regions. A third protein region included peptide 1, peptide 2 and peptide 3 ("peptide 1+2+3"); in this case, a glycine residue was inserted "*ex novo*" between peptide 1 and peptides 2 and 3. The I165P substitution in peptide 2 was maintained.

EXAMPLE 3

Cloning and expression of SLO protein fragments as His or GST fusions

[247] SLO protein fragments were expressed as His fusions as shown in FIG. 5. SLO protein fragments were expressed as GST fusions as shown in FIG. 6.

EXAMPLE 4

MALDI-TOF analysis of GAS25 6Xhis fragments

[248] PAGE analysis of the 6xhis fusions of the three GAS SLO fragments demonstrated a discrepancy between the expected and the observed molecular weights of the recombinant polypeptides (FIG. 15). Peptide 1, which has an expected molecular weight (MW) of 9,300.1 Dalton, had an observed MW of about 25,000 Dalton. Peptide 2+3 has an expected MW of 10,277 Dalton but an observed MW of 15,000-16,000 Dalton. Peptide 1+2+3 has an expected MW of 18,370 Dalton and an observed MW of about 30,000 Dalton. The three polypeptides were therefore subjected to MALDI-TOF analysis, which confirmed the expected molecular weights. The results are shown in FIGS. 16-20.

[249] FIG. 16 shows the MALDI-TOF analysis of peptide 1 in solution. The peak at 9170,226 corresponds to peptide without the Met residue (9300 dalton –131 dalton of Met = 9170 dalton). Others peaks correspond to the markers used for instrument calibration. Removal of the Met residue at N terminal of proteins expressed in *E. coli* is very common if the second amino acid is small and hydrophobic.

[250] FIG. 17 shows the MALDI-TOF analysis of peptide 2+3 in solution. The peak at 10097,523 correspond to peptide 2+3 without the Met residue (10,227 dalton –131 dalton of Met = 10,096 dalton). Others peaks correspond to the markers used for instrument calibration.

[251] FIG. 18 shows the MALDI-TOF analysis of peptide 2+3 digested with trypsin. Proteins digested with trypsin show peak profiles that are characteristic of each peptide (finger printing). Each peak corresponds to a fragment of the digested protein. Peptide 2+3 digested with trypsin shows the following characteristic peaks:

- 1,090.549 aa 22-30
- 1,218.637 aa 22-31 and aa 21-30

- 1,659.847 aa 6-20
- 2,025.968 aa 34-52
- 2,154.129 aa 33-52
- 2,282.229 aa 35-52
- 2,770.448 aa 68-90

[252] FIG. 19 shows the MALDI-TOF analysis of peptide 1+2+3 in solution. The peak at 18,236.998 corresponds to peptide 1+2+3 peptide without the Met residue (18,370 dalton −131 dalton of Met = 18,239 dalton). Other peaks are either degradation products or *E. coli* contaminants.

[253] FIG. 20 shows the MALDI-TOF analysis of peptide 1+2+3 digested with trypsin. The fingerprinting technique reveals many peaks belonging to peptide 1+2+3:

- 1090.542 aa 96-104
- 1247.550 aa 80-90
- 1695.641 aa 127-141
- 1932,830 aa 73-90
- 2025.859 aa 108-126
- 2153.924 aa 107-126
- 2852.121 aa 8-33

EXAMPLE 5

In vivo protection experiments

[254] Mice were immunized with different SLO fragments and challenged with the M1 strain of GAS. Groups of 10-20 mice were immunized with 20 mg of the recombinant protein at days 0, 21, and 35. Mice of negative control groups were immunized either with GST

alone or with *E. coli* contaminants, depending on the version of the GAS recombinant protein used. Blood samples were taken two weeks after the third immunization. A few days after that, immunized mice were challenged intranasally with 108 cfu (50 ml) of an M1 GAS strain (3348 strain). Survival of mice was monitored for a 10-14 day period and compared to survival of negative control groups. Immune sera obtained from the different groups were tested for immunogenicity with the entire SLO recombinant protein (Western blot analysis).

[255] The results are shown in Table 1. These results demonstrate that the SLO fragments confer protection against GAS infection when used as immunogens.

Table 1.

Antigen*	Experiment	n° mice	Survival (%)	Negative control survival (%)	Sera reactivity against SLO in WB
25_1 his	1	10	**40**	30	NO
25_1 his	2	10	**70**	20	NO
25_1 his	3	20	**60**	30	YES
25_2 his	1	10	**40**	30	YES
25_2 his	2	10	**70**	20	YES
25_2 his urea	1	10	**50**	60	NT
25_2 his urea	3	20	**40**	15	NT
25_tot his	1	10	**50**	30	YES
25_tot his	2	10	**60**	20	YES
25_tot_his	3	20	**50**	30	YES
25_1 GST	1	10	**80**	20	YES
25_1 GST	2	10	**30**	40	YES
25_1 GST	3	20	**85**	10	YES
25_2 GST	1	10	**80**	20	YES
25_2 GST	2	10	**60**	40	YES
25_2 GST	3	20	**55**	10	YES
25_tot GST	1	10	**90**	20	YES
25_tot GST	2	10	**40**	40	YES
25_tot GST	3	20	**40**	10	YES

* 25_1 his (SEQ ID NO:8); 25_2 his (SEQ ID NO:10); 25_tot his (SEQ ID NO:12); 25_1 GST (SEQ ID NO:14); 25_2 GST (SEQ ID NO:16); and 25_tot (SEQ ID NO:18).

CLAIMS

1. A composition comprising an active agent selected from the group consisting of:

(a) a *Streptococcus pyogenes* streptolysin O (SLO) antigen consisting essentially of an amino acid sequence selected from the group consisting of:

(i) SEQ ID NO:1;

(ii) SEQ ID NO:2;

(iii) SEQ ID NO:3;

(iv) SEQ ID NO:2 covalently attached to SEQ ID NO:3;

(v) an amino acid sequence consisting essentially of (1) SEQ ID NO:1; (2) a glycine residue covalently attached to the amino acid sequence SEQ ID NO:1; (3) the amino acid sequence SEQ ID NO:2 covalently attached to the glycine; and (4) the amino acid sequence SEQ ID NO:3 covalently attached to the amino acid sequence SEQ ID NO:2;

(vi) SEQ ID NO:8;

(vii) SEQ ID NO:10;

(vii) amino acids 2-82 of SEQ ID NO:10;

(viii) SEQ ID NO:12;

(ix) amino acids 4-156 of SEQ ID NO:12;

(x) SEQ ID NO:14;

(xi) SEQ ID NO:16; and

(xii) SEQ ID NO:18;

wherein the SLO antigen is non-toxic;

(b) a nucleic acid molecule which encodes the SLO antigen; and

(c) an antibody which specifically binds to the SLO antigen.

2. The composition of claim 1 wherein the active agent is the SLO antigen and wherein the SLO antigen is monomeric.

3. The composition of claim 1 or claim 2 further comprising a GAS40 antigen.

4. The composition of claim 1 or claim 2 further comprising a GAS57 antigen.

5. The composition of any of claims 1-4 further comprising an antigen which is useful in a pediatric vaccine.

6. The composition of any of claims 1-4 further comprising an antigen which is useful in a vaccine for elderly or immunocompromised individuals.

7. The composition of any of claims 1-6 further comprising an adjuvant.

8. The composition of any of claims 1-7 wherein the active agent is the SLO antigen and the SLO antigen is coupled to a carrier protein.

9. The composition of claim 8 wherein the carrier protein is selected from the group consisting of a bacterial toxin, a bacterial toxoid, a *N. meningitidis* outer membrane protein, a heat shock protein, a pertussis protein, *H. influenzae* protein D, a cytokine, a lymphokine, a hormone, a growth factor, *C. difficile* toxin A, *C. difficile* toxin B, and an iron-uptake protein.

10. A method of making a vaccine for inducing immunity against *S. pyogenes* comprising combining the active agent of any of claims 1-9 with a pharmaceutically acceptable carrier, wherein the active agent is the SLO antigen or the nucleic acid molecule.

11. The method claim 10 wherein the active agent is the SLO antigen and the SLO antigen is made by a method comprising:

(a) culturing a host cell comprising an expression vector which encodes the SLO antigen; and

(b) recovering the SLO antigen.

12. A method of inducing immunity against *S. pyogenes* comprising administering to an individual an effective amount of the composition of any of claims 1-9, wherein the active agent is the SLO antigen or the nucleic acid molecule.

13. A method of treating a *S. pyogenes* infection comprising administering to an individual an effective amount of the composition of any of claims 1, 3, 4, 5, 6, or 7, wherein the active agent is the antibody.

14. Use of the active agent of any of claims 1-9 in the manufacture of a medicament for inducing immunity against *S. pyogenes* infection, wherein the active agent is the SLO antigen or the nucleic acid molecule.

15. Use of the active agent of any of claims 1, 3, 4, 5, 6, or 7 in the manufacture of a medicament for treating a *S. pyogenes* infection, wherein the active agent is the antibody.

FIG. 1

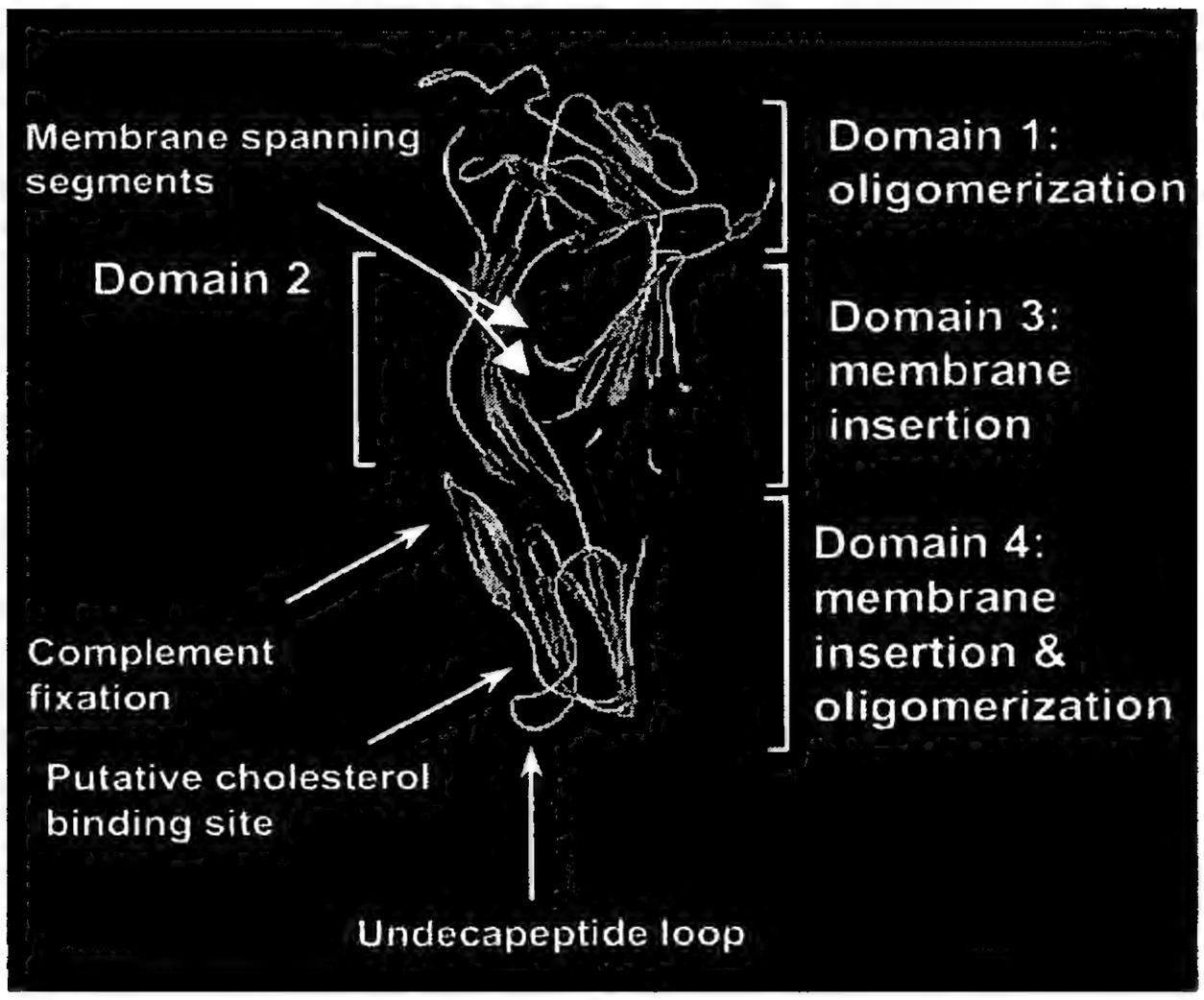

Membrane spanning segments
Domain 2
Complement fixation
Putative cholesterol binding site
Undecapeptide loop
Domain 1: oligomerization
Domain 3: membrane insertion
Domain 4: membrane insertion & oligomerization

FIG. 2

gi|3401988|pdb|1PFO| S Perfringolysin O
Length=500

Score = 592 bits (1525), Expect = 9e-170, Method: Composition-based stats.
Identities = 311/471 (66%), Positives = 384/471 (81%), Gaps = 0/471 (0%)

```
Query  100  KSEEDHTEEINDKIYSLNYNELEVLAKNGETIENFVPKEGVKKADKFIVIERKKKNINTT  159
            K   D  + I+  I SL+YN  EVLA NG+ IE+FVPKEG K  +KFIV+ER+K+++ T+
Sbjct  29   KDITDKNQSIDSGISSLSYNRNEVLASNGDKIESFVPKEGKKAGNKFIVVERQKRSLTTS  88

Query  160  PVDISIIDSVTDRTYPAALQLANKGFTENKPDAVVTKRNPQKIHIDLPGMGDKATVEVND  219
            PVDISIIDSV DRTYP ALQLA+K   EN+P  ++ KR P  I+IDLPG+  + +++V+D
Sbjct  89   PVDISIIDSVNDRTYPGALQLADKALVENRPTILMVKRKPININIDLPGLKGENSIKVDD  148

Query  220  PTYANVSTAIDNLVNQWHDNYSGGNTLPARTQYTESMVYSKSQIEAALNVNSKILDGTLG  279
            PTY  VS AID LV++W++ YS  +TLPARTQY+ESMVYSKSQI +ALNVN+K+L+ +LG
Sbjct  149  PTYGKVSGAIDELVSKWNEKYSSTHTLPARTQYSESMVYSKSQISSALNVNAKVLENSLG  208

Query  280  IDFKSISKGEKKVMIAAYKQIFYTVSANLPNNPADVFDKSVTFKELQRKGVSNEAPPLFV  339
            +DF +++  EKKVMI AYKQIFYTVSA+LP NP+D+FD SVTF +L++KGVSNEAPPL V
Sbjct  209  VDFNAVANNEKKVMILAYKQIFYTVSADLPKNPSDLFDDSVTFNDLKQKGVSNEAPPLMV  268

Query  340  SNVAYGRTVFVKLETSSKSNDVEAAFSAALKGTDVKTNGKYSDILENSSFTAVVLGGDAA  399
            SNVAYGRT++VKLET+S S DV+AAF A +K TD+K + +Y DI ENSSFTAVVLGGDA
Sbjct  269  SNVAYGRTIYVKLETTSSSKDVQAAFKALIKNTDIKNSQQYKDIYENSSFTAVVLGGDAQ  328

Query  400  EHNKVVTKDFDVIRNVIKDNATFSRKNPAYPISYTSVFLKNNKIAGVNNRTEYVETTSTE  459
            EHNKVVTKDFD IR VIKDNATFS KNPAYPISYTSVFLK+N +A V+N+T+Y+ETTSTE
Sbjct  329  EHNKVVTKDFDEIRKVIKDNATFSTKNPAYPISYTSVFLKDNSVAAVHNKTDYIETTSTE  388

Query  460  YTSGKINLSHQGAYVAQYEILWDEINYDDKGKEVITKRRWDNNWYSKTSPFSTVIPLGAN  519
            Y+ GKINL H GAYVAQ+E+ WDE++YD +G EV+T + WD N+  KT+ +STVIPL AN
Sbjct  389  YSKGKINLDHSGAYVAQFEVAWDEVSYDKEGNEVLTHKTWDGNYQDKTAHYSTVIPLEAN  448

Query  520  SRNIRIMARECTGLAWEWWRKVIDERDVKLSKEINVNISGSTLSPYGSITY  570
            +RNIRI ARECTGLAWEWWR VI E DV L+  INV+I G+TL P  SITY
Sbjct  449  ARNIRIKARECTGLAWEWWRDVISEYDVPLTNNINVSIWGTTLYPGSSITY  499
```

FIG. 3

Signal sequence ■ Domain 1 □ Domain 2 ■ Domain 3 ■ Domain 4

- aa 1-31 (blue line) correspond to the predicted signal sequence
- aa 1-99 are not present in the Perfringolysin O sequence (see homology above)
- Peptides underlined in black were identified by surfome analysis in SF370-M1 (pep1 and pep2) and in 3650-M6 (pep3) strains

  - pep1: **36-QNTASTETTTTNEQPKPESSELTTEK-61**
  - pep2: **155-NINTTPVDISIIDSVTDR-172**
  - pep3: **450-TEYVETTSTEY-460**

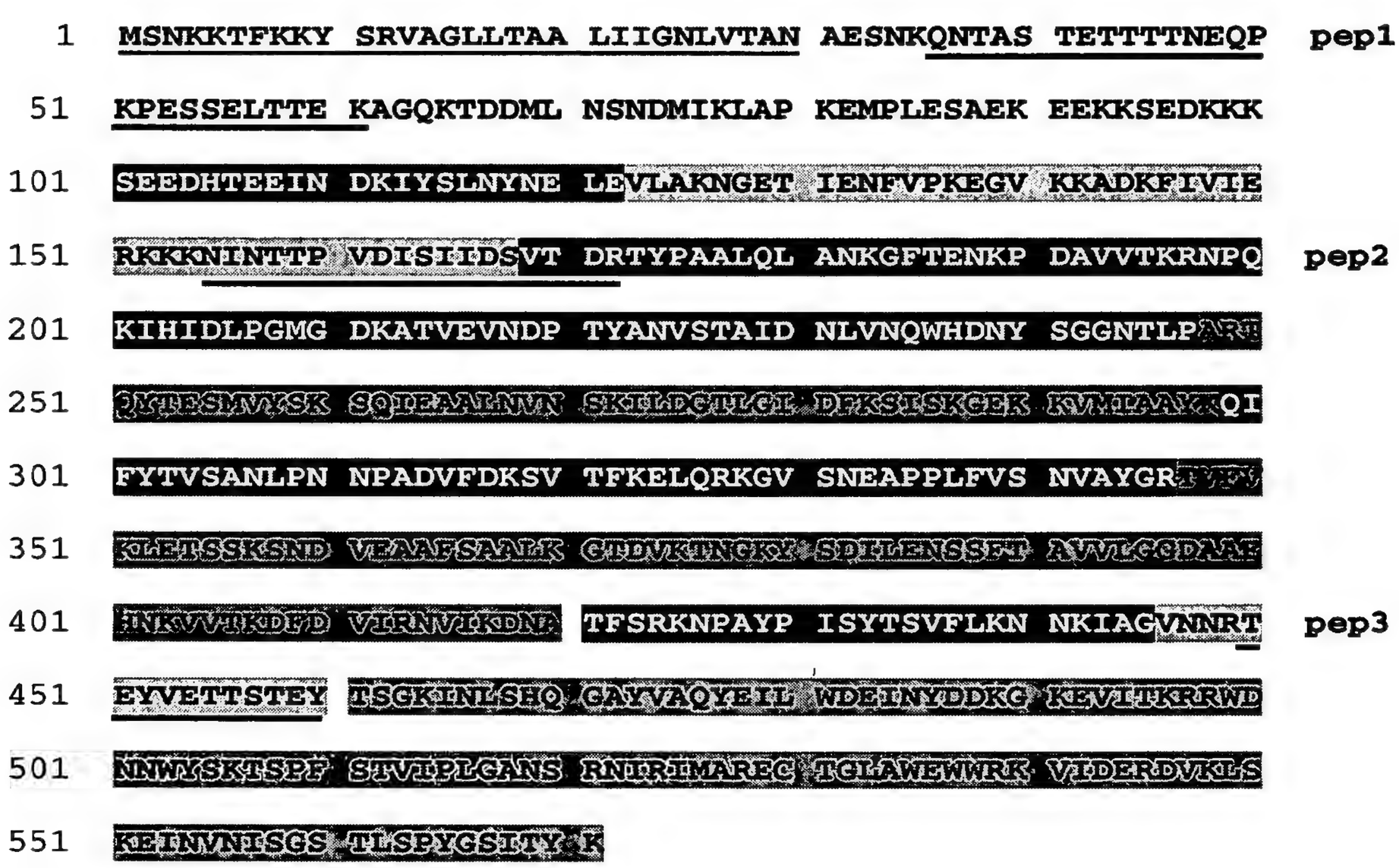


FIG. 4

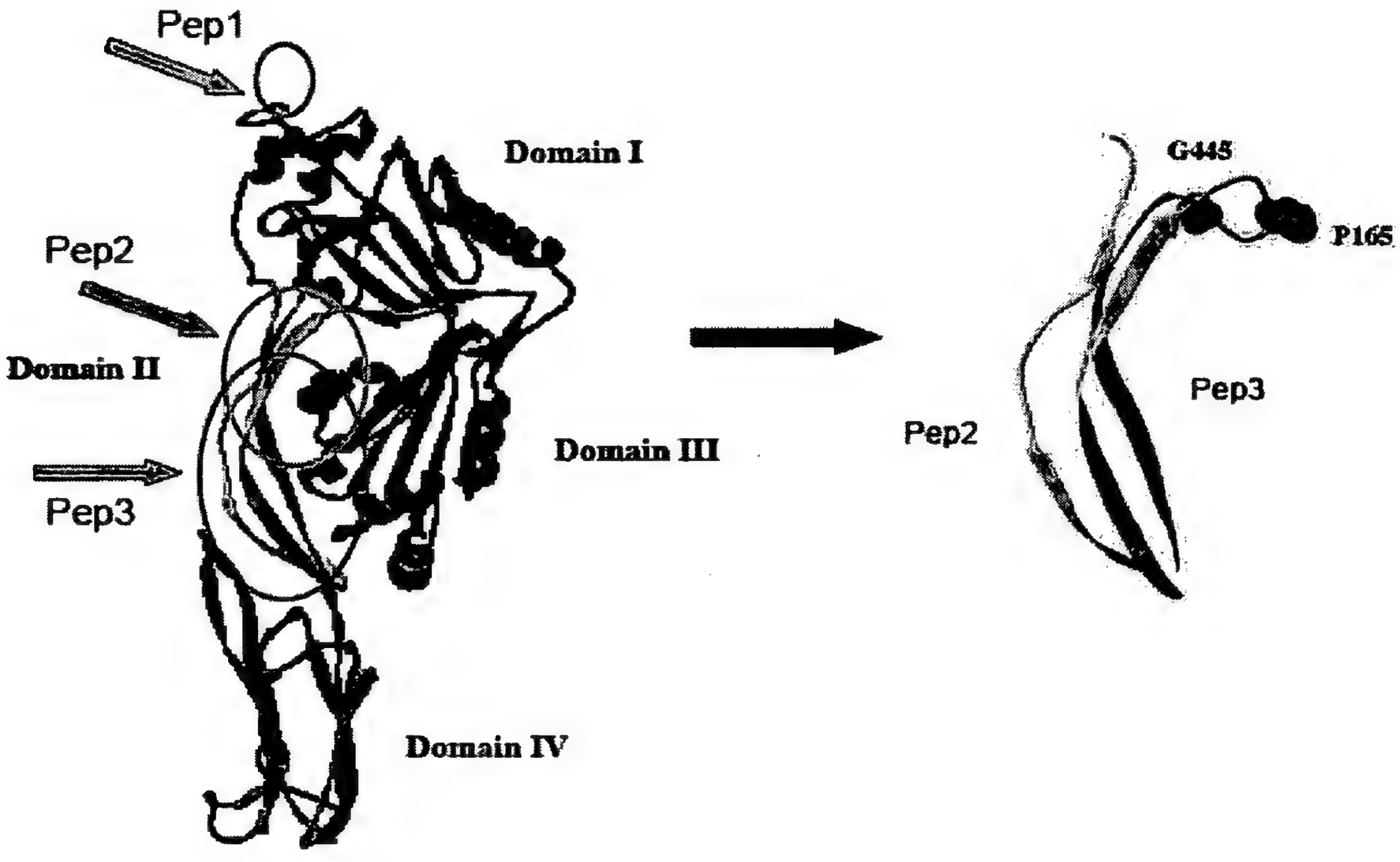

Pep1
Domain I
Pep2
Domain II
Pep3
Domain III
Domain IV
G445
P165
Pep2
Pep3

FIG. 5

■ Amino acids derived from the cloning vector DNA sequences from vector

25_1 in Pet 21b(+)
(249 bp)

SEQ ID NO:7

ATG**GCTAGC**GAATCGAACAAACAAAACACTGCTAGTACAGAAACCACAACGACAAATGAGCAAC
CAAAGCCAGAAAGTAGTGAGCTAACTACTGAAAAAGCAGGTCAGAAAACGGATGATATGCTTAA
CTCTAACGATATGATTAAGCTTGCTCCCAAAGAAATGCCACTAGAATCTGCAGAAAAAGAAGAA
AAAAAGTCAGAAGACAAAAAAAAGAGCGAA**CTCGAG**CACCACCACCACCACCAC**TGA**

SEQUENCE 82 AA; MW 9300.1

SEQ ID NO:8

Met A S E S N K Q N T A S T E T T T T N E Q P K P E S S E L T T E K A G Q K T D D
M L N S N D M I K L A P K E M P L E S A E K E E K K S E D K K K S E L E H H H H H H
H Stop

25_2 in Pet 21b(+)
(273 bp)

SEQ ID NO:9

ATGGTACTTGCTAAAAATGGTGAAACCATTGAAAATTTTGTTCCTAAAGAAGGCGTTAAGAAAG
CTGATAAATTTATTGTCATTGAAAGAAAGAAAAAAAATATCAACACTACACCAGTCGATATTTCC
CCTATTGACTCTGGTGTCAATAACAGAACTGAATACGTTGAAACAACATCTACCGAGTACACTAG
TGGAAAAATTAACCTGTCTCATCAAGGCGCGTATGTTGCTCAATATGAAATC**CTCGAG**CACCAC
CACCACCACCAC**TGA**

SEQUENCE 90 AA; 10227 MW

SEQ ID NO:10

Met VLAKNGETIENFVPKEGVKKADKFIVIERKKKNINTTPVDI
SPIDSGVNNRTEYVETTSTEYTSGKINLSHQGAYVAQYEILE
HHHHHHH Stop

25_tot (1+2+3)in Pet 21b(+)
(495 bp)

SEQ ID NO:11

ATG**GCTAGC**GAATCGAACAAACAAAACACTGCTAGTACAGAAACCACAACGACAAATGAGCAAC
CAAAGCCAGAAAGTAGTGAGCTAACTACTGAAAAAGCAGGTCAGAAAACGGATGATATGCTTAA
CTCTAACGATATGATTAAGCTTGCTCCCAAAGAAATGCCACTAGAATCTGCAGAAAAAGAAGAA
AAAAAGTCAGAAGACAAAAAAAAGAGCGAAGGTGTACTTGCTAAAAATGGTGAAACCATTGAAA
ATTTTGTTCCTAAAGAAGGCGTTAAGAAAGCTGATAAATTTATTGTCATTGAAAGAAAGAAAAAA
AATATCAACACTACACCAGTCGATATTTCCCCTATTGACTCTGGTGTCAATAACAGAACTGAATA
CGTTGAAACAACATCTACCGAGTACACTAGTGGAAAAATTAACCTGTCTCATCAAGGCGCGTATG
TTGCTCAATATGAAATC**CTCGAG**CACCACCACCACCACCAC**TGA**

SEQUENCE 164 AA; MW 18370.3 GGT= glycine CCT=proline

SEQ ID NO:12

Met A S ESNKQNTASTETTTTNEQPKPESSELTTEKAGQKTDD
MLNSNDMIKLAPKEMPLESAEKEEKKSEDKKKSE**G**VLAKNG
ETIENFVPKEGVKKADKFIVIERKKKNINTTPVDIS**P**IDS**G**VN
NRTEYVETTSTEYTSGKINLSHQGAYVAQYEILEHHHHHHH
Stop

FIG. 6

■ GST vector-derived ■ Aminoacids derived from the cloning vector

25_1 in Pgex
(924 bp)

SEQ ID NO:13

ATGTCCCCTATACTAGGTTATTGGAAAATTAAGGGCCTTGTGCAACCCACTCGACTTCTTTTGG
AATATCTTGAAGAAAAATATGAAGAGCATTTGTATGAGCGCGATGAAGGTGATAAATGGCGAAA
CAAAAAGTTTGAATTGGGTTTGGAGTTTCCCAATCTTCCTTATTATATTGATGGTGATGTTAAA
TTAACACAGTCTATGGCCATCATACGTTATATAGCTGACAAGCACAACATGTTGGGTGGTTGTC
CAAAAGAGCGTGCAGAGATTTCAATGCTTGAAGGAGCGGTTTTGGATATTAGATACGGTGTTTC
GAGAATTGCATATAGTAAAGACTTTGAAACTCTCAAAGTTGATTTTCTTAGCAAGCTACCTGAA
ATGCTGAAAATGTTCGAAGATCGTTTATGTCATAAAACATATTTAAATGGTGATCATGTAACCC
ATCCTGACTTCATGTTGTATGACGCTCTTGATGTTGTTTTATACATGGACCCAATGTGCCTGGA
TGCGTTCCCAAAATTAGTTTGTTTTAAAAAACGTATTGAAGCTATCCCACAAATTGATAAGTAC
TTGAAATCCAGCAAGTATATAGCATGGCCTTTGCAGGGCTGGCAAGCCACGTTTGGTGGTGGCG
ACCATCCTCCAAAATCGGATCTGGTTCCGCGT**CATATGGCTAGC**GAATCGAACAAACAAAACAC
TGCTAGTACAGAAACCACAACGACAAATGAGCAACCAAAGCCAGAAAGTAGTGAGCTAACTACT
GAAAAAGCAGGTCAGAAAACGGATGATATGCTTAACTCTAACGATATGATTAAGCTTGCTCCCA
AAGAAATGCCACTAGAATCTGCAGAAAAAGAAGAAAAAAAGTCAGAAGACAAAAAAAAGAGCGA
A**CTCGAG**CATCACCATCACCATCAC

SEQUENCE 307 AA; MW 35585.6

SEQ ID NO:14

Met S P I L G Y W K I K G L V Q P T R L L L E Y L E E K Y E E H L Y E R D E G D K
W R N K K F E L G L E F P N L P Y Y I D G D V K L T Q S M A I I R Y I A D K H N M
L G G C P K E R A E I S M L E G A V L D I R Y G V S R I A Y S K D F E T L K V D F
L S K L P E M L K M F E D R L C H K T Y L N G D H V T H P D F M L Y D A L D V V
L Y M D P M C L D A F P K L V C F K K R I E A I P Q I D K Y L K S S K Y I A W P L
Q G W Q A T F G G G D H P P K S D L V P R H Met A S E S N K Q N T A S T E T T T
T N E Q P K P E S S E L T T E K A G Q K T D D M L N S N D M I K L A P K E M P L
E S A E K E E K K S E D K K K S E L E H H H H H H Stop

25_2 in pGEX
(948 bp)

SEQ ID NO:15

ATGTCCCCTATACTAGGTTATTGGAAAATTAAGGGCCTTGTGCAACCCACTCGACTTCTTTTGG
AATATCTTGAAGAAAAATATGAAGAGCATTTGTATGAGCGCGATGAAGGTGATAAATGGCGAAA
CAAAAAGTTTGAATTGGGTTTGGAGTTTCCCAATCTTCCTTATTATATTGATGGTGATGTTAAA
TTAACACAGTCTATGGCCATCATACGTTATATAGCTGACAAGCACAACATGTTGGGTGGTTGTC
CAAAAGAGCGTGCAGAGATTTCAATGCTTGAAGGAGCGGTTTTGGATATTAGATACGGTGTTTC
GAGAATTGCATATAGTAAAGACTTTGAAACTCTCAAAGTTGATTTTCTTAGCAAGCTACCTGAA
ATGCTGAAAATGTTCGAAGATCGTTTATGTCATAAAACATATTTAAATGGTGATCATGTAACCC
ATCCTGACTTCATGTTGTATGACGCTCTTGATGTTGTTTTATACATGGACCCAATGTGCCTGGA
TGCGTTCCCAAAATTAGTTTGTTTTAAAAAACGTATTGAAGCTATCCCACAAATTGATAAGTAC
TTGAAATCCAGCAAGTATATAGCATGGCCTTTGCAGGGCTGGCAAGCCACGTTTGGTGGTGGCG
ACCATCCTCCAAAATCGGATCTGGTTCCGCGT**CATATG**GTACTTGCTAAAAATGGTGAAACCAT
TGAAAATTTTGTTCCTAAAGAAGGCGTTAAGAAAGCTGATAAATTTATTGTCATTGAAAGAAAGA
AAAAAAATATCAACACTACACCAGTCGATATTTCCCGTATTGACTCTGGTGTCAATAACAGAACT
GAATACGTTGAAACAACATCTACCGAGTACACTAGTGGAAAAATTAACCTGTCTCATCAAGGCGC
GTATGTTGCTCAATATGAAATC**CTCGAG**CATCACCATCACCATCAC***

SEQUENCE 315 AA; 36513 MW

SEQ ID NO:16

MetSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDK
WRNKKFELGLEFPNLPYYIDGDVKLTQSMAIIRYIADKHNM
LGGCPKERAEISMLEGAVLDIRYGVSRIAYSKDFETLKVDF
LSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALDVV
LYMDPMCLDAFPKLVCFKKRIEAIPQIDKYLKSSKYIAWPL
QGWQATFGGGDHPPKSDLVPRHMetVLAKNGETIENFVPKE
GVKKADKFIVIERKKKNINTTPVDISPIDSGVNNRTEYVETT
STEYTSGKINLSHQGAYVAQYEILEHHHHHH Stop

25_tot (1+2+3)in pGEX
(1170 bp)

SEQ ID NO:17

HGTCCCCTATACTAGGTTATTGGAAAATTAAGGGCCTTGTGCAACCCACTCGACTTCTTTTGG
AATATCTTGAAGAAAAATATGAAGAGCATTTGTATGAGCGCGATGAAGGTGATAAATGGCGAAA
CAAAAAGTTTGAATTGGGTTTGGAGTTTCCCAATCTTCCTTATTATATTGATGGTGATGTTAAA
TTAACACAGTCTATGGCCATCATACGTTATATAGCTGACAAGCACAACATGTTGGGTGGTTGTC
CAAAAGAGCGTGCAGAGATTTCAATGCTTGAAGGAGCGGTTTTGGATATTAGATACGGTGTTTC
GAGAATTGCATATAGTAAAGACTTTGAAACTCTCAAAGTTGATTTTCTTAGCAAGCTACCTGAA
ATGCTGAAAATGTTCGAAGATCGTTTATGTCATAAAACATATTTAAATGGTGATCATGTAACCC
ATCCTGACTTCATGTTGTATGACGCTCTTGATGTTGTTTTATACATGGACCCAATGTGCCTGGA
TGCGTTCCCAAAATTAGTTTGTTTTAAAAAACGTATTGAAGCTATCCCACAAATTGATAAGTAC
TTGAAATCCAGCAAGTATATAGCATGGCCTTTGCAGGGCTGGCAAGCCACGTTTGGTGGTGGCG
ACCATCCTCCAAAATCGGATCTGGTTCCGCGT**CATATGGCTAGC**GAATCGAACAAACAAAACAC
TGCTAGTACAGAAACCACAACGACAAATGAGCAACCAAAGCCAGAAAGTAGTGAGCTAACTACT
GAAAAAGCAGGTCAGAAAACGGATGATATGCTTAACTCTAACGATATGATTAAGCTTGCTCCCA
AAGAAATGCCACTAGAATCTGCAGAAAAAGAAGAAAAAAAGTCAGAAGACAAAAAAAAGAGCGA
AGGTGTACTTGCTAAAAATGGTGAAACCATTGAAAATTTTGTTCCTAAAGAAGGCGTTAAGAAA
GCTGATAAATTTATTGTCATTGAAAGAAAGAAAAAAAATATCAACACTACACCAGTCGATATTTC
CCCTATTGACTCTGGTGTCAATAACAGAACTGAATACGTTGAAACAACATCTACCGAGTACACTA
GTGGAAAAATTAACCTGTCTCATCAAGGCGCGTATGTTGCTCAATATGAAATC**CTCGAG**CATCA
CCATCACCATCAC ---

SEQUENCE 389 AA; MW 44655.8

SEQ ID NO:18

MSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDKW
RNKKFELGLEFPNLPYYIDGDVKLTQSMAIIRYIADKHNML
GGCPKERAEISMLEGAVLDIRYGVSRIAYSKDFETLKVDFL
SKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALDVVL
YMDPMCLDAFPKLVCFKKRIEAIPQIDKYLKSSKYIAWPLQ
GWQATFGGGDHPPKSDLVPR HMet AS ESNKQNTASTETTTT
NEQPKPESSELTTEKAGQKTDDMLNSNDMIKLAPKEMPLE
SAEKEEKKSEDKKKSEGVLAKNGETIENFVPKEGVKKADK
FIVIERKKKNINTTPVDISPIDSGVNNRTEYVETTSTEYTSGK
INLSHQGAYVAQYEI LEHHHHHH Stop

FIG. 7

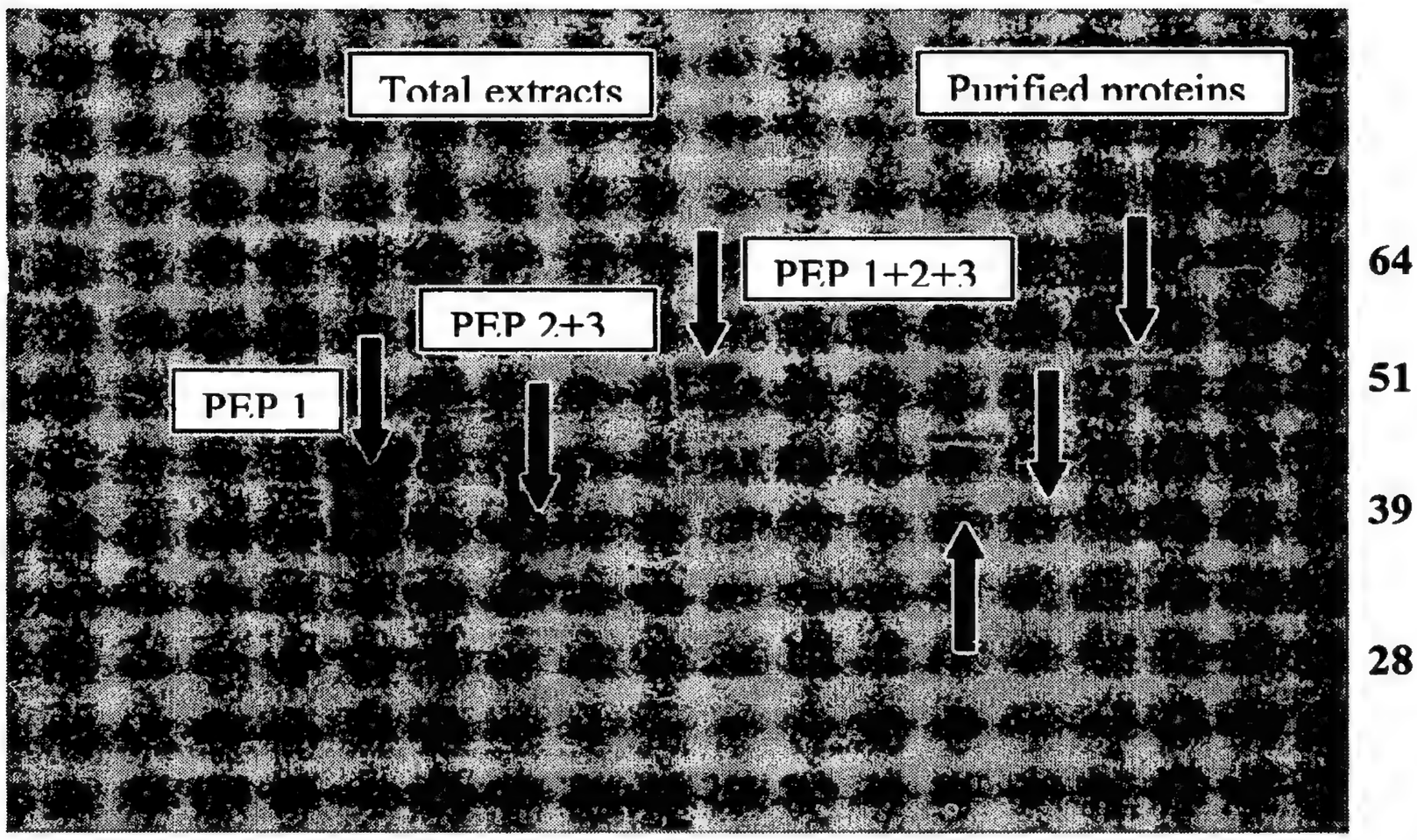

pGEX fusion proteins
Total extracts
Purified proteins
PEP 1+2+3
PEP 2+3
PEP 1
64
51
39
28

FIG. 8

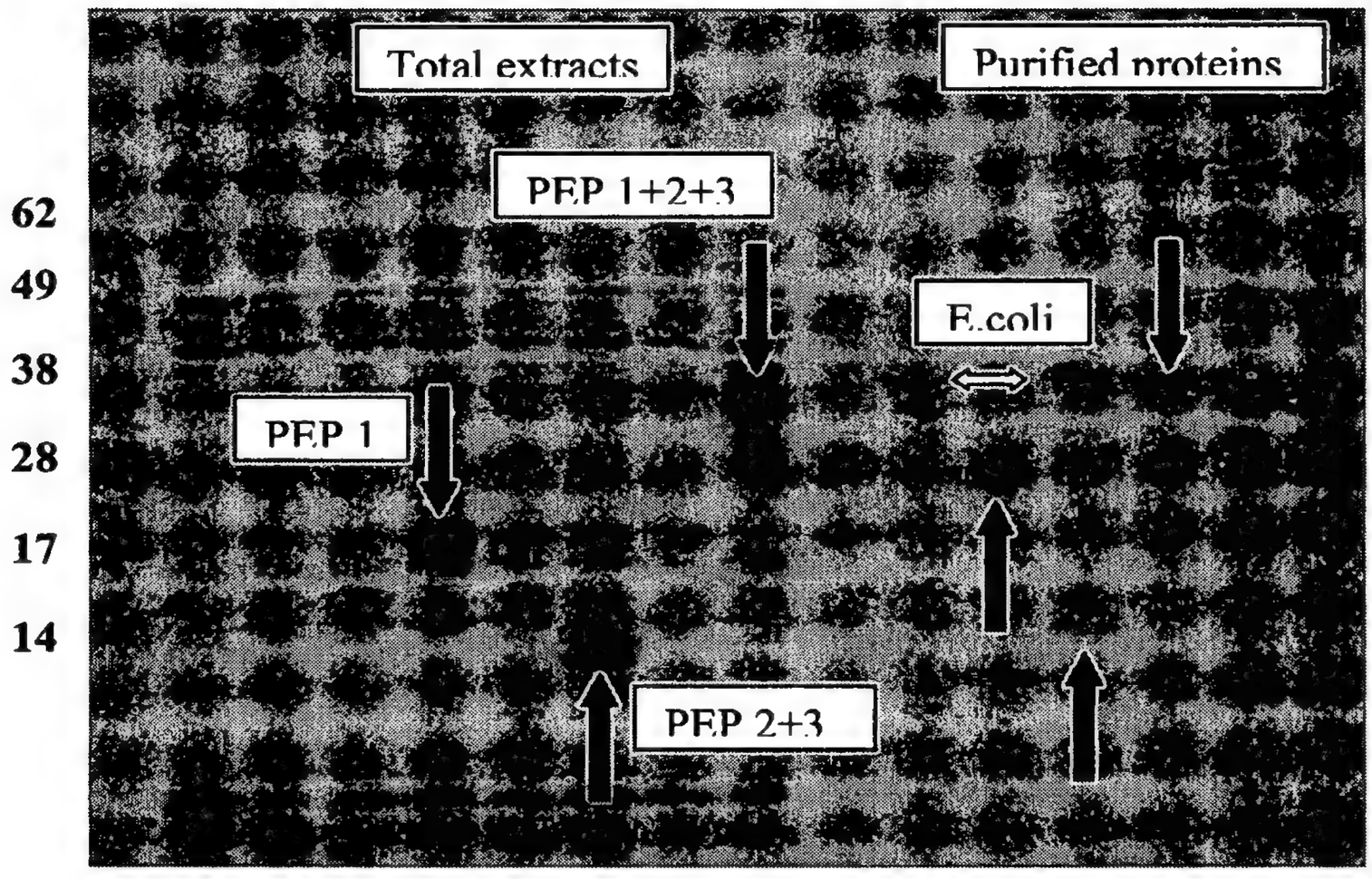

6Xhis fusion proteins
Total extracts
Purified proteins
PEP 1+2+3
E.coli
PEP 1
PEP 2+3
62
49
38
28
17
14

FIG. 9

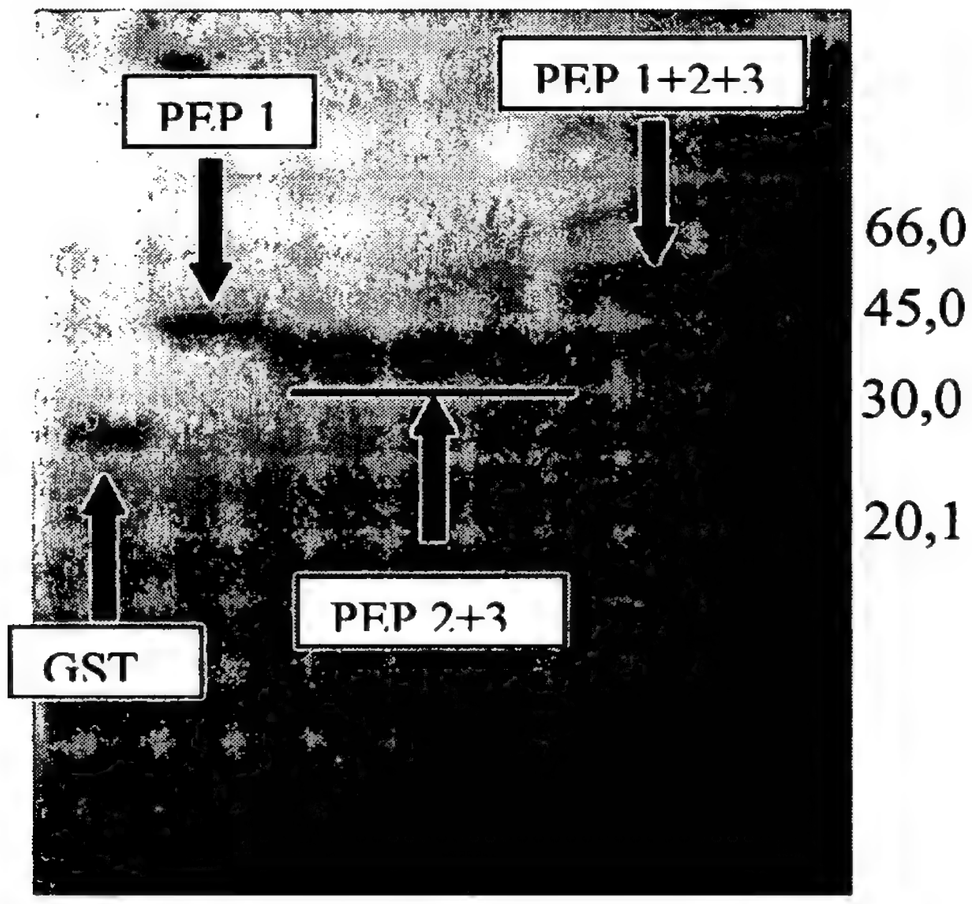


FIG. 10

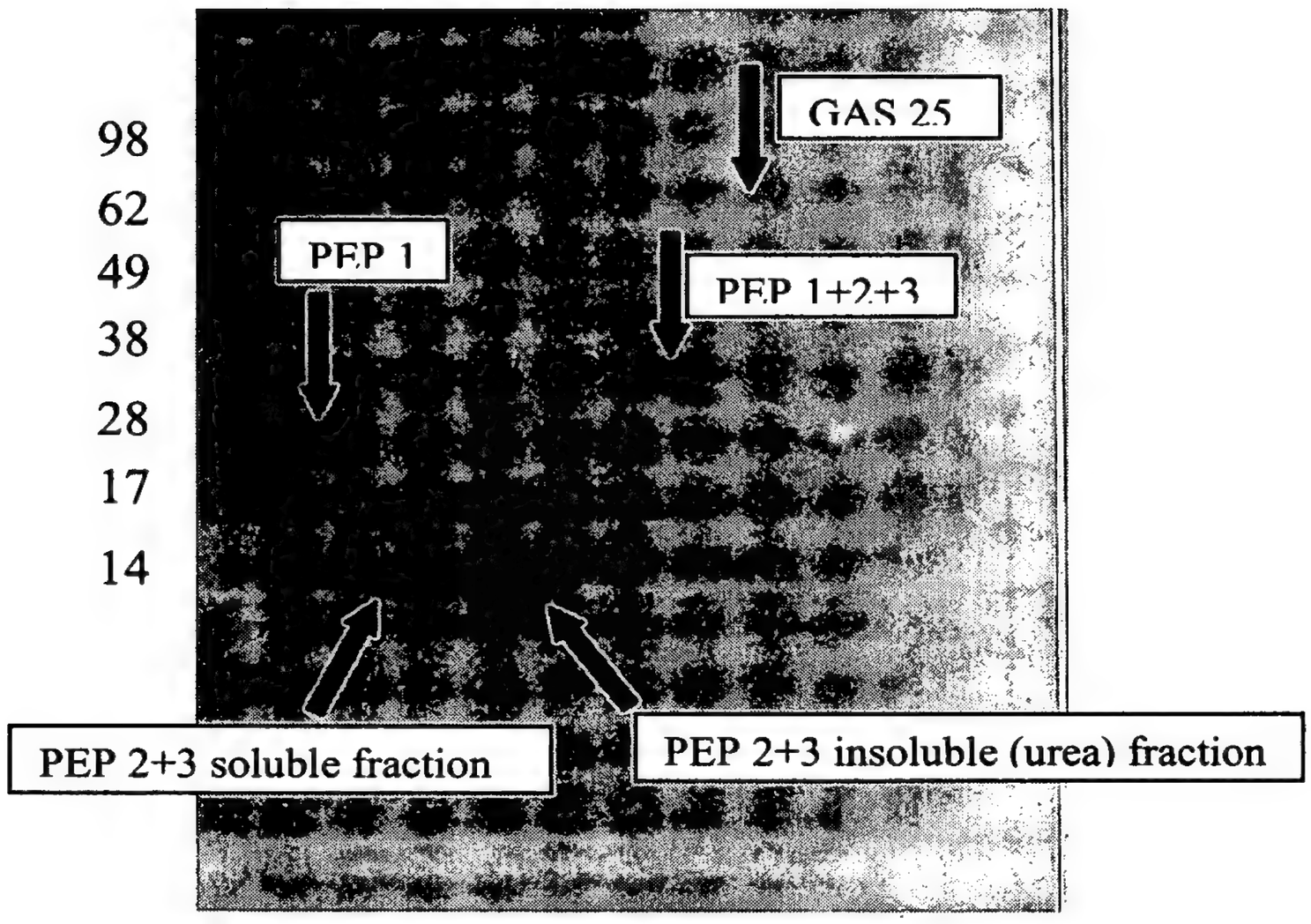
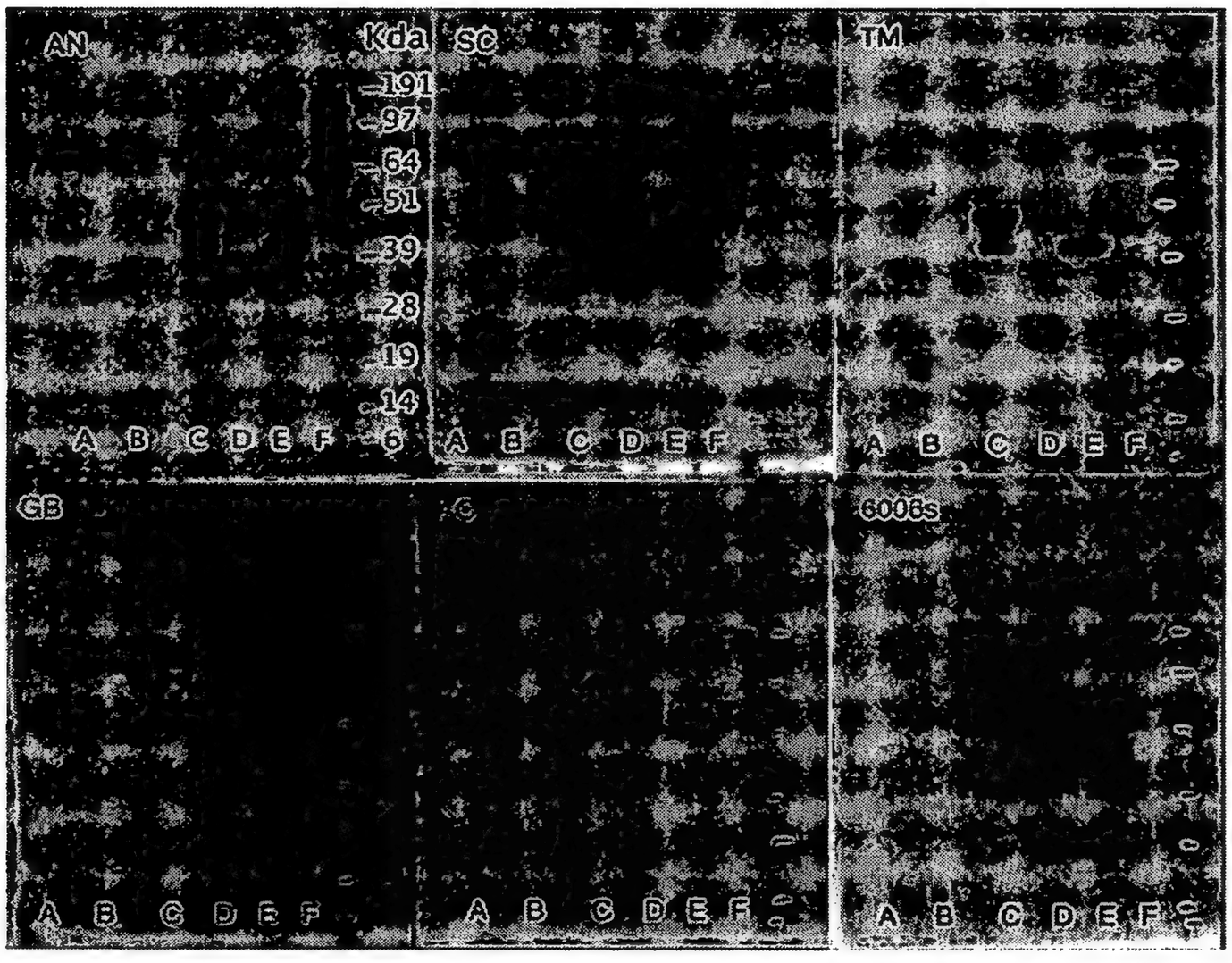

6Xhis fusion proteins
98
62
49
38
28
17
14
GAS 25
PEP 1
PEP 1+2+3
PEP 2+3 soluble fraction
PEP 2+3 insoluble (urea) fraction

FIG. 11



A: E.coli contaminants control

B: GST protein control

C: PEP 1+2+3

D: PEP 2+3

E: PEP 1

F: GAS 25

FIG. 12

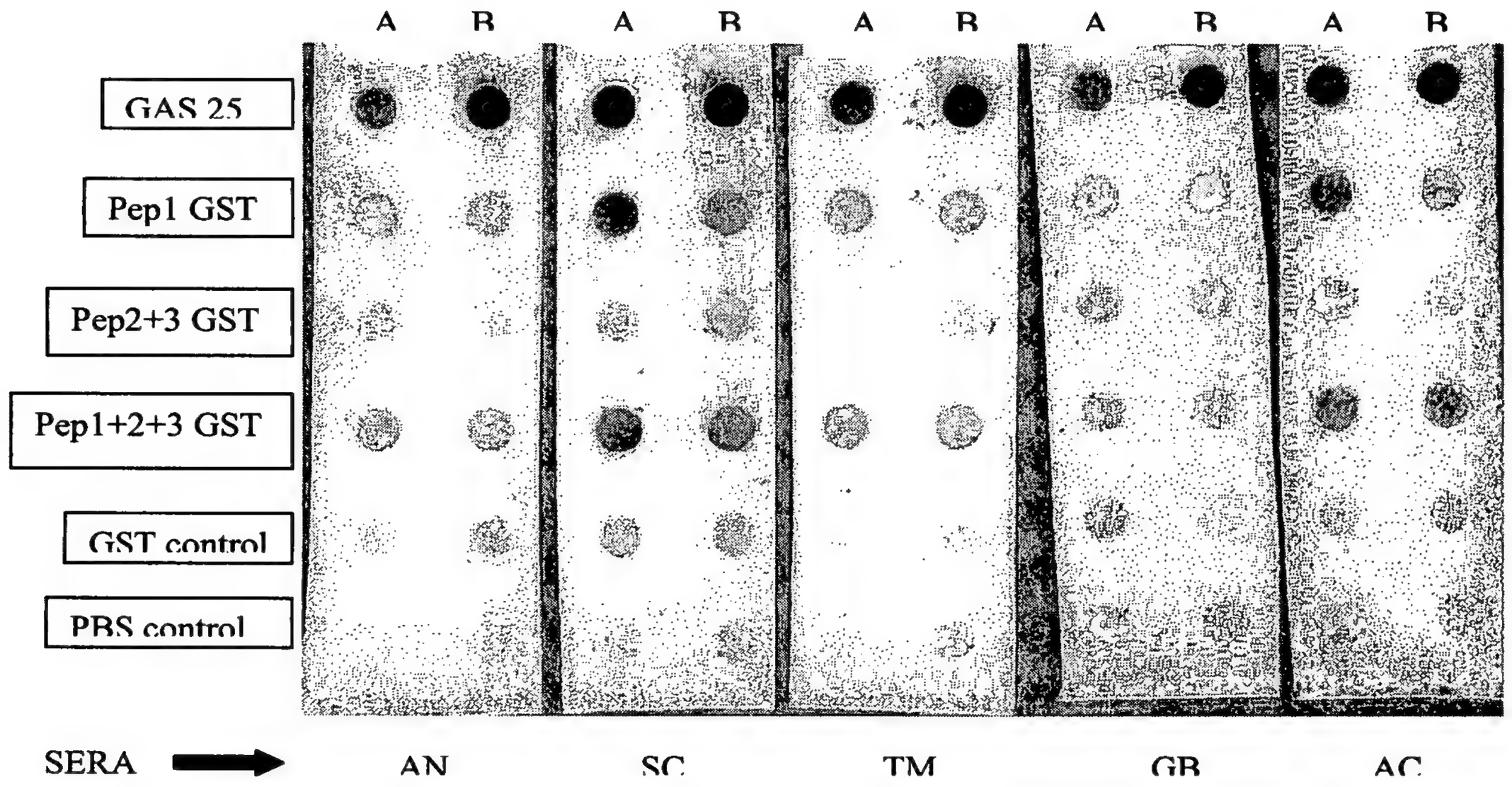
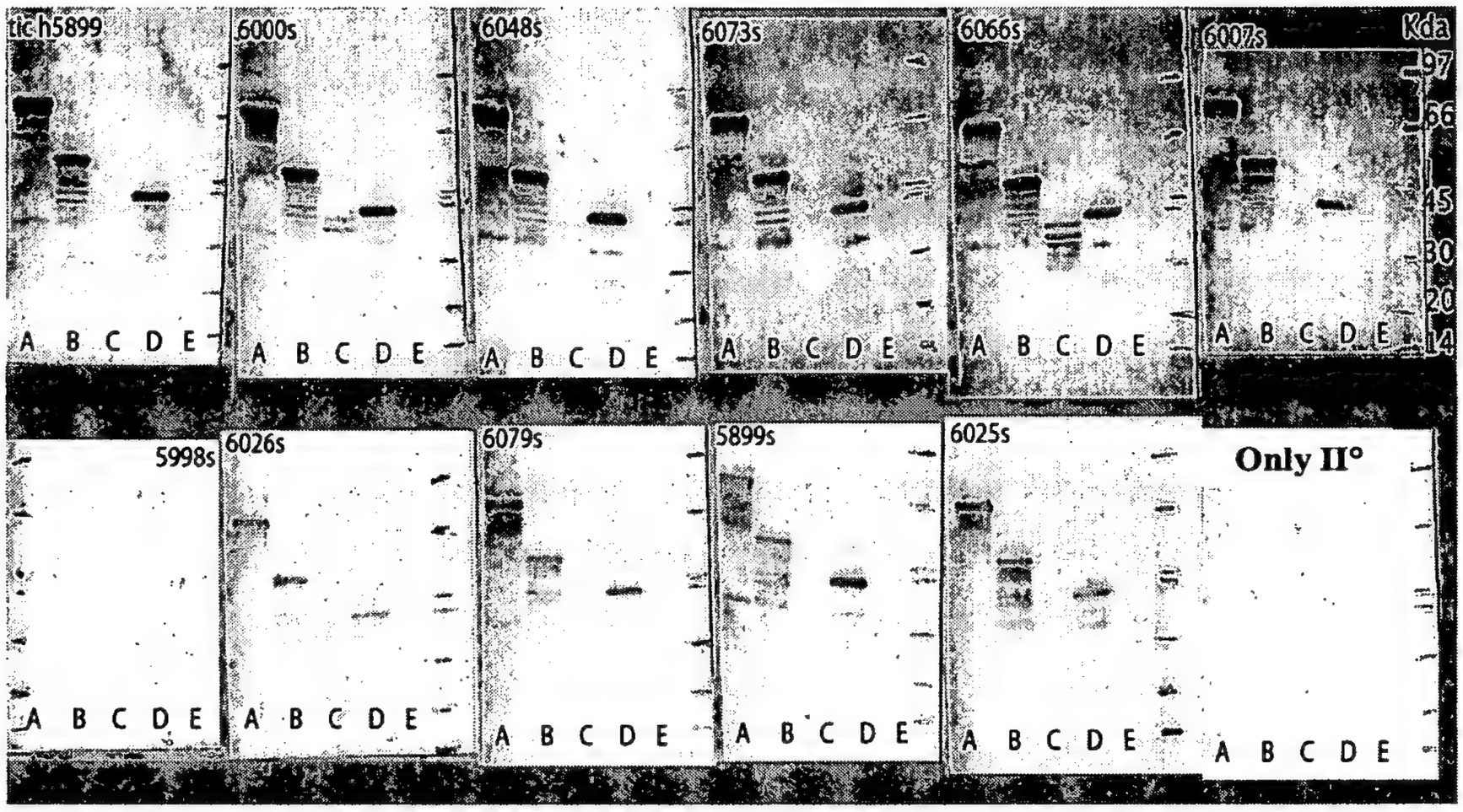

A
B
A
B
A
B
A
B
A
B
GAS 25
Pep1 GST
Pep2+3 GST
Pep1+2+3 GST
GST control
PBS control
SERA
AN
SC
TM
GB
AC

FIG. 13



A: GAS 25 B: PEP 1+2+3 C: PEP 2+3 D: PEP1 E: GST

FIG. 14

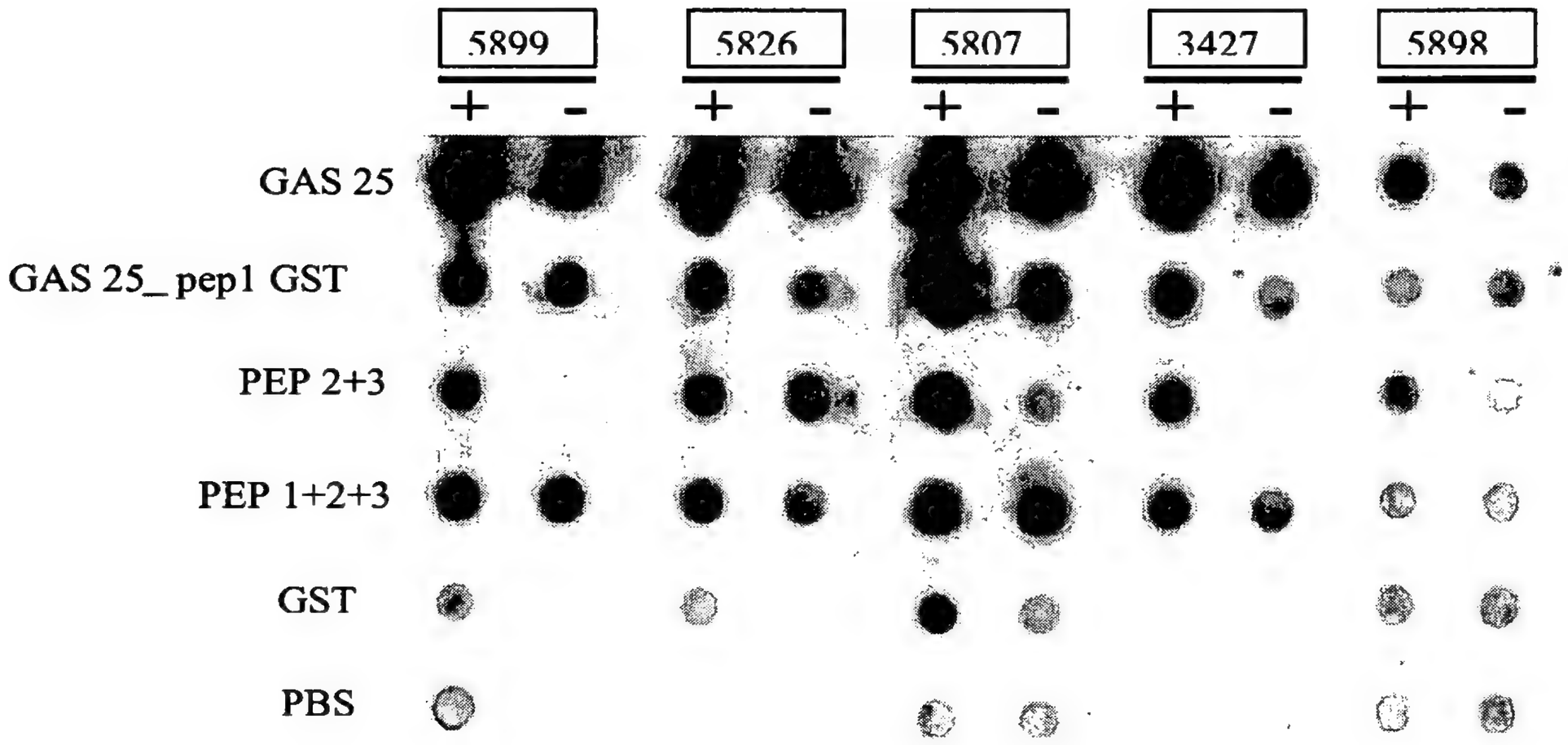

5899
5826
5807
3427
5898
+ - + - + - + - + -
GAS 25
GAS 25_ pep1 GST
PEP 2+3
PEP 1+2+3
GST
PBS

FIG. 15

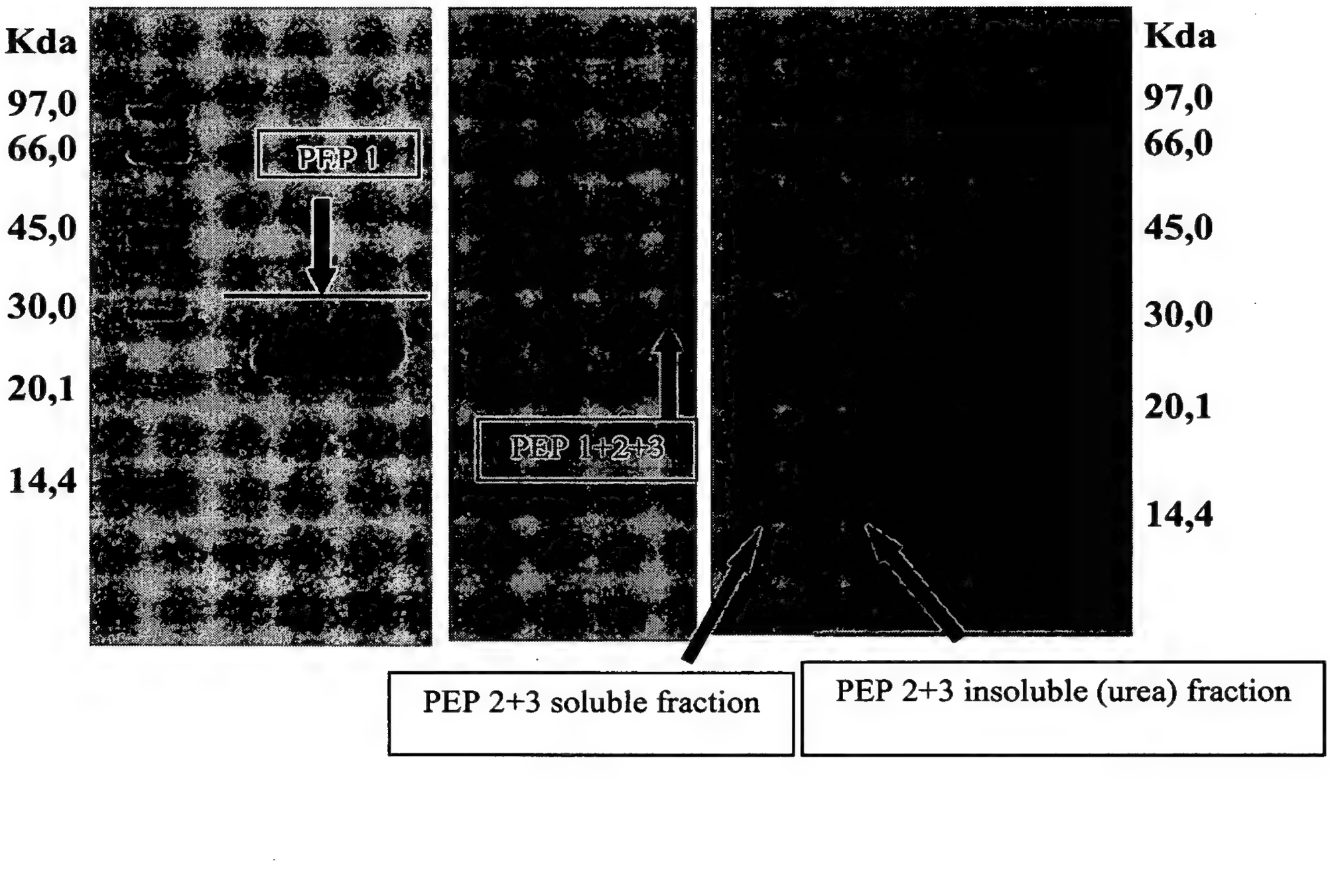

6Xhis fusion proteins
Kda
97,0
66,0
45,0
30,0
20,1
14,4
PEP 1
PEP 1+2+3
Kda
97,0
66,0
45,0
30,0
20,1
14,4
PEP 2+3 soluble fraction
PEP 2+3 insoluble (urea) fraction

FIG. 16

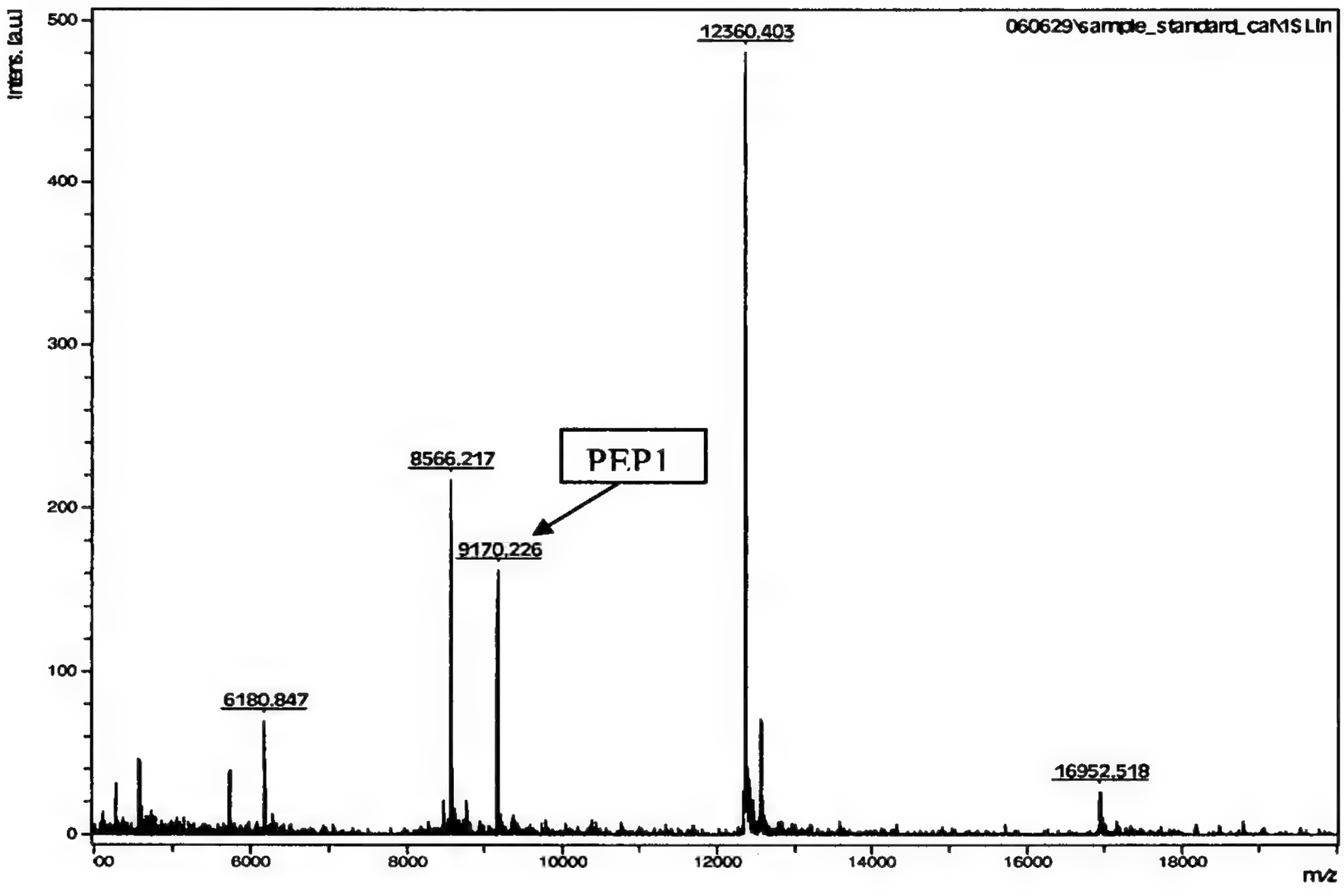

Intens. [a.u.]
500
400
300
200
100
0
12360.403
060629\sample_standard_caM1S L1n
8566.217
PEP1
9170.226
6180.847
16952.518
00
6000
8000
10000
12000
14000
16000
18000
m/z

FIG. 17

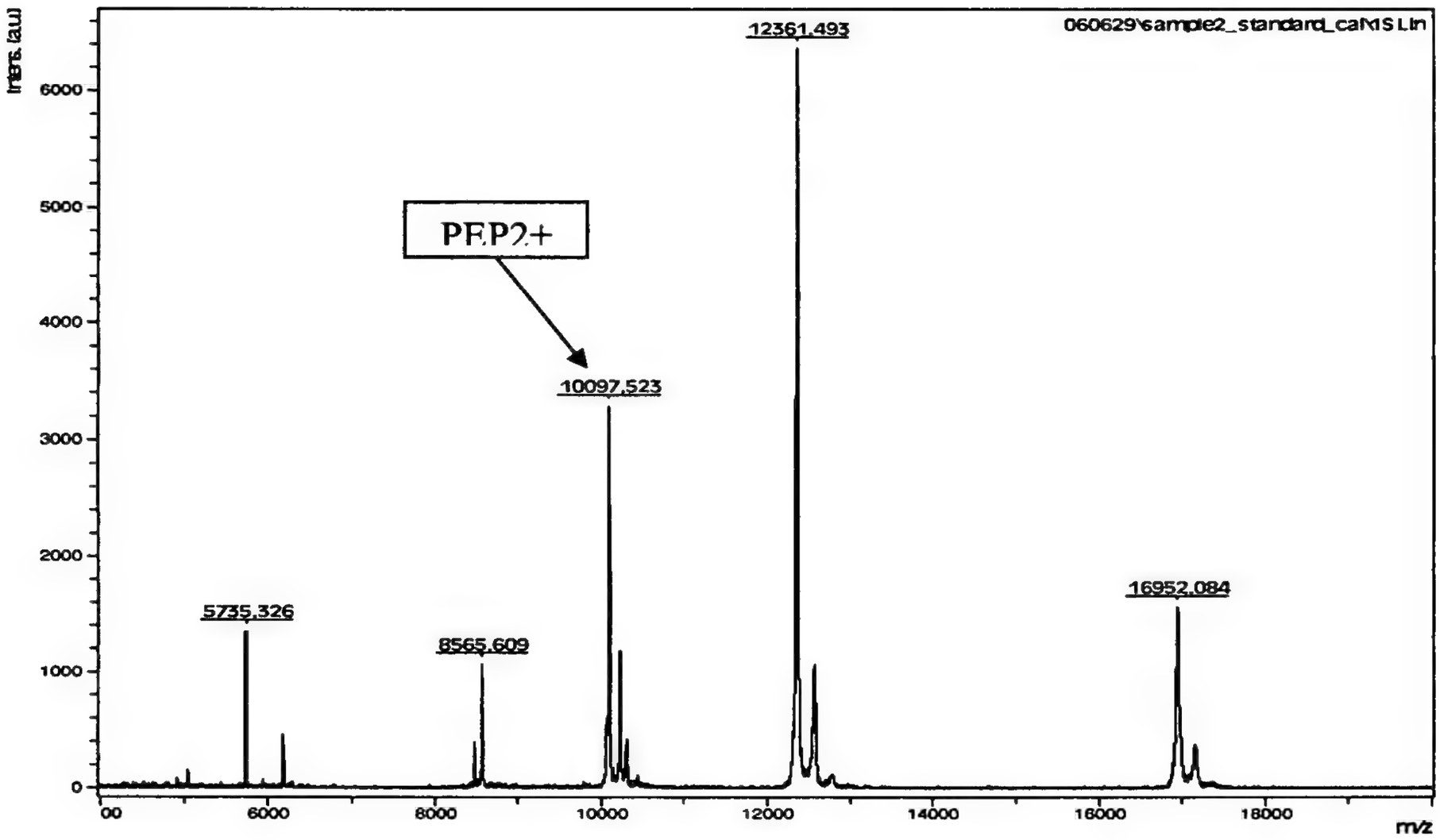

060629\sample2_standard_calMS.Lin
Intens. [a.u.]
6000
5000
4000
3000
2000
1000
0
PEP2+
12361.493
10097.523
5735.326
8565.609
16952.084
00
6000
8000
10000
12000
14000
16000
18000
m/z

FIG. 18

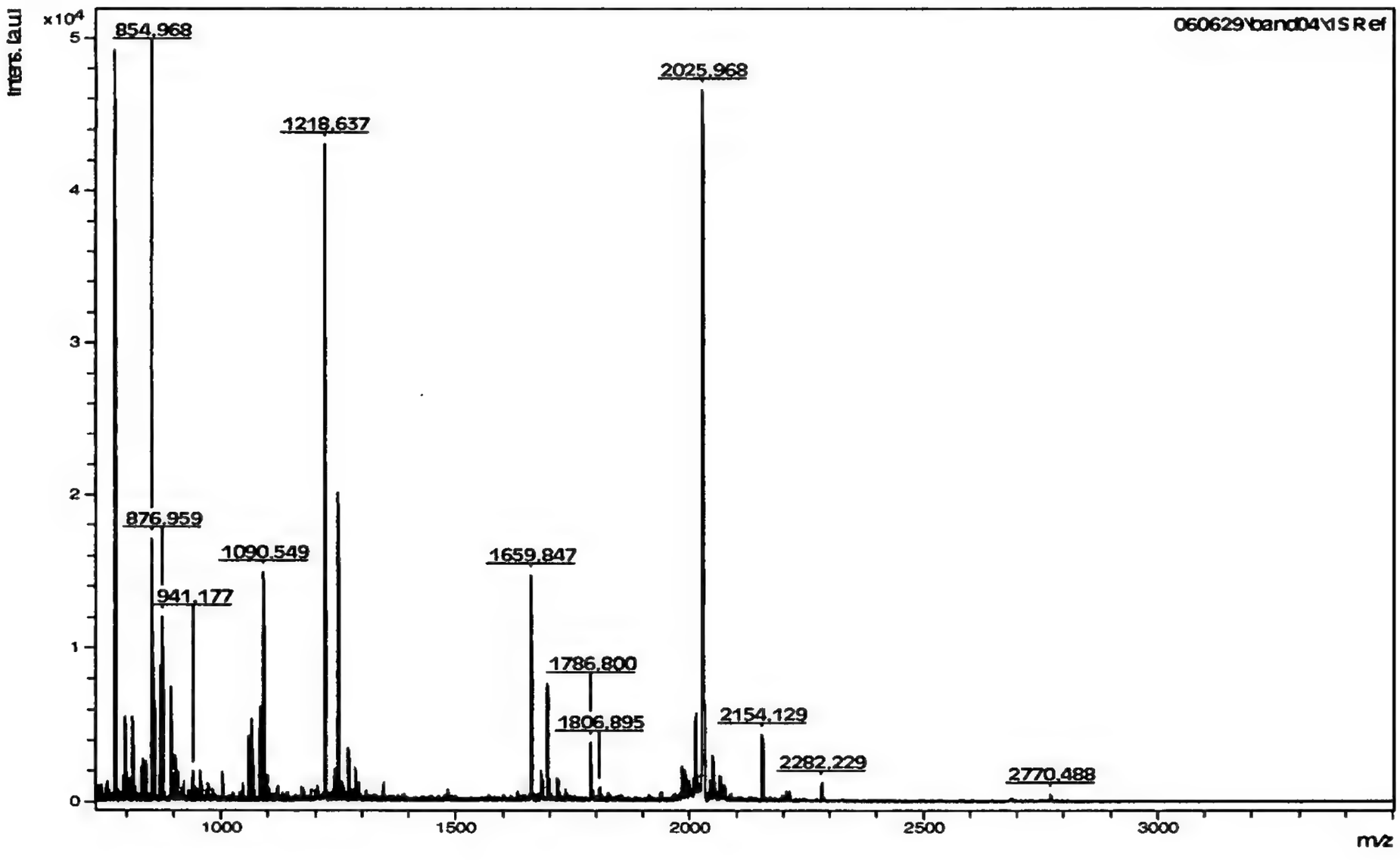

Intens. [a.u.]
x10^4
5
4
3
2
1
0
060629\band04\1SRef
854.968
2025.968
1218.637
876.959
1090.549
1659.847
941.177
1786.800
1806.895
2154.129
2282.229
2770.488
1000
1500
2000
2500
3000
m/z

FIG. 19

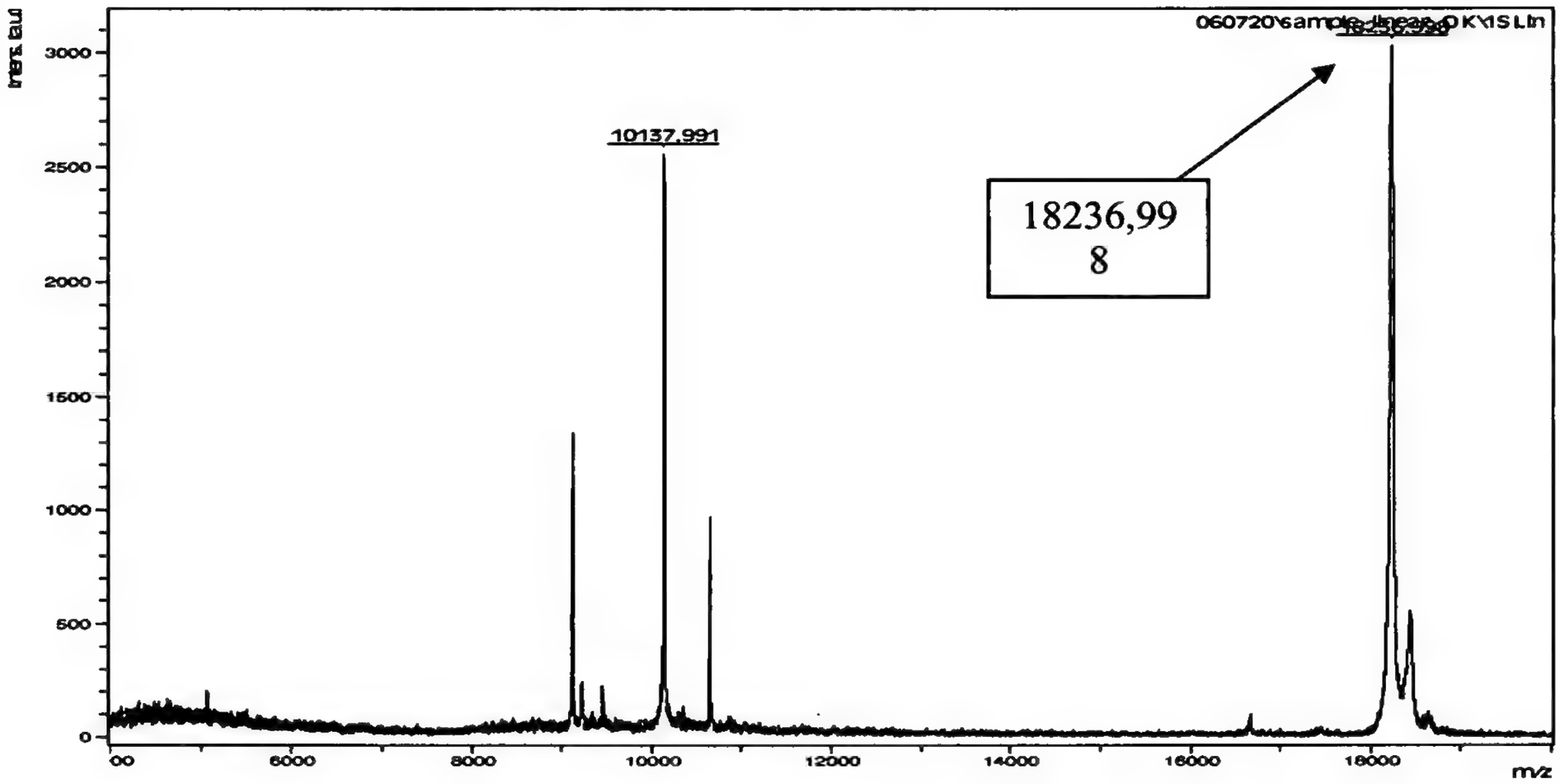
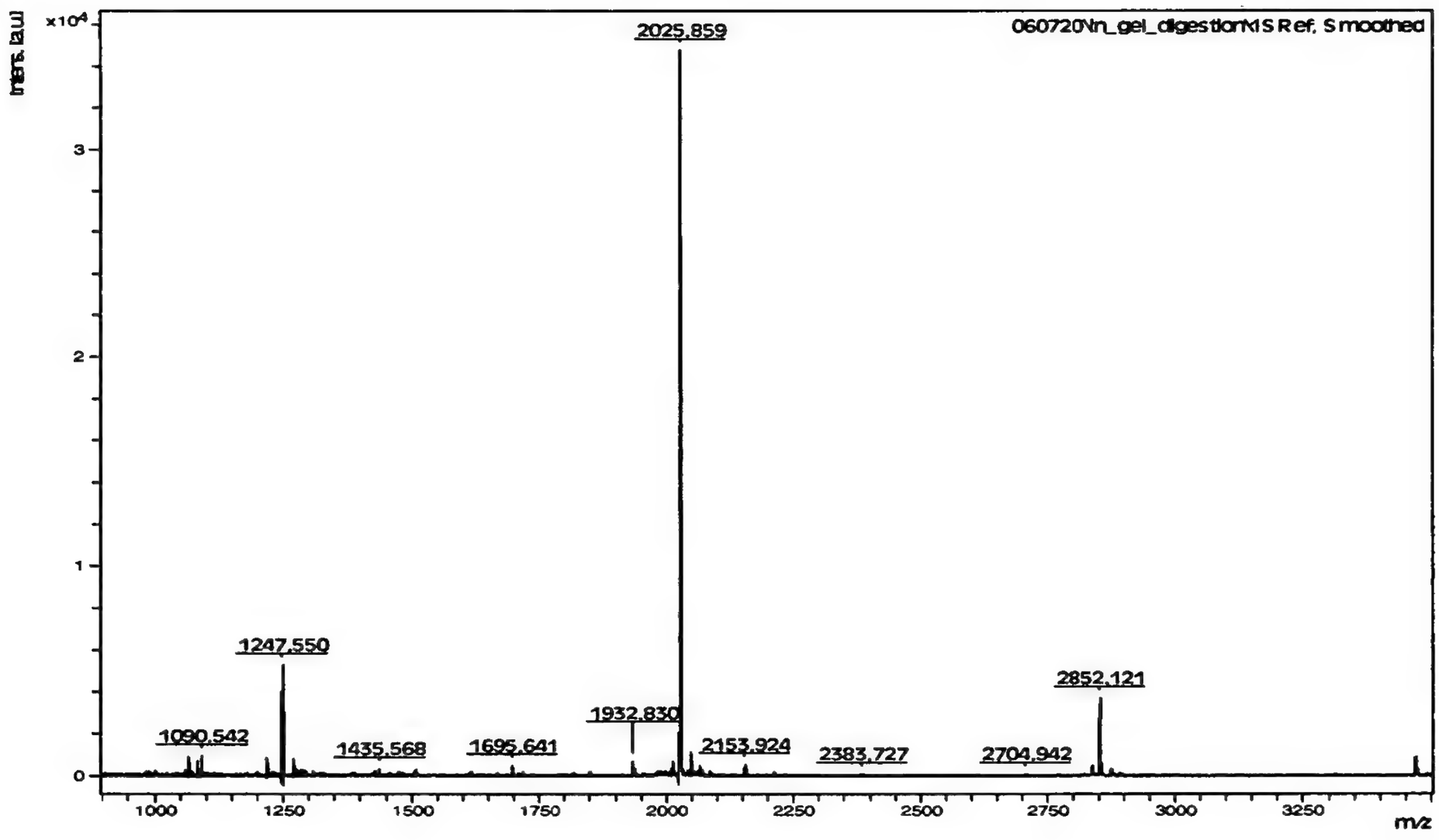

060720\sample_linear_OK\1SLin
10137.991
18236,99
8
Inten. [a.u.]
3000
2500
2000
1500
1000
500
0
00
6000
8000
10000
12000
14000
16000
18000
m/z

FIG. 20


x10^4
Intens. [a.u.]
3
2
1
0
2025.859
060720\tn_gel_digestion\MS Ref, Smoothed
1247.550
1090.542
1435.568
1695.641
1932.830
2153.924
2383.727
2704.942
2852.121
1000
1250
1500
1750
2000
2250
2500
2750
3000
3250
m/z

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(71) Applicant *(for all designated States except US)*: **NOVARTIS AG** [US/US]; c/o Novartis Vaccines And Diagnostics Inc., P.O. Box 8097, Emeryville, CA 94622-8097 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SCARSELLI, Maria** [IT/IT]; C/o Novartis Vaccines & Diagnostics, Inc., P.O. Box 8097, Emeryville, CA 94608-8097 (US). **BENSI, Giuliano** [IT/IT]; c/o Novartis Vaccines & Diagnostics, Inc., P.O. Box 8097, Emeryville, CA 94608-8097 (US). **GRANDI, Guido** [IT/IT]; Chiron S.r.l., Via Fiorentina 1, I-53100 (IT).

(74) Agent: **HEMMENDINGER, Lisa, M.**; BANNER & WITCOFF, LTD., 1100 13th Street, N.W., Suite 1200, Washington, DC 20005-4051 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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[Continued on next page]

(54) Title: IMMUNOGENIC AND THERAPEUTIC COMPOSITIONS FOR *STREPTOCOCCUS PYOGENES*

(57) Abstract: Compositions for preventing and/or treating S. *pyogenes* infection which comprise one or more active agents. The active agents are SLO antigens, nucleic acid molecules encoding the SLO antigens, and/or antibodies which selectively bind to the SLO antigens.

FIG. 3

Signal sequence ■ Domain 1 □ Domain 2 ■ Domain 3 ■ Domain 4

- aa 1-31 (blue line) correspond to the predicted signal sequence
- aa 1-99 are not present in the Perfringolysin O sequence (see homology above)
- Peptides underlined in black were identified by surfome analysis in SF370-M1 (pep1 and pep2) and in 3650-M6 (pep3) strains
  - ➢ pep1: **36-QNTASTETTTTNEQPKPESSELTTEK-61**
  - ➢ pep2: **155-NINTTPVDISIIDSVTDR-172**
  - ➢ pep3: **450-TEYVETTSTEY-460**

```
  1  MSNKKTFKKY SRVAGLLTAA LIIGNLVTAN AESNKQNTAS TETTTTNEQP  pep1
 51  KPESSELTTE KAGQKTDDML NSNDMIKLAP KEMPLESAEK EEKKSEDKKK
101  SEEDHTEEIN DKIYSLNYNE LEVLAKNGET IENFVPKEGV KKADKFIVIE
151  RKKKNINTTP VDISIIDSVT DRTYPAALQL ANKGFTENKP DAVVTKRNPQ  pep2
201  KIHIDLPGMG DKATVEVNDP TYANVSTAID NLVNQWHDNY SGGNTLPARG
251  SKTFSMVYSK SQLEAALNVN SKILDGTLGL DFKSISKGEK KVMIAAFKQI
301  FYTVSANLPN NPADVFDKSV TFKELQRKGV SNEAPPLFVS NVAYGRTVFV
351  KLETSSKSND VEAAFSAALK GTDVKTNGKY SDILENSSFT AVVLGGDAAE
401  INKVVTKDFD VIRNVLKDNA TFSRKNPAYP ISYTSVFLKN NKIAGVNNRT  pep3
451  EYVETTSTEY TSCKINLSHQ GAYVAQVEIL WDEINYDDKG KEVITKRRWD
501  NNWYSKTSPF STVIPLGANS RNIRIMAREC TGLAWEWWRK VIDERDVKLS
551  KEINVNISGS TLSPYGSITY K
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European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

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— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2007/022838

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/315 A61K39/09

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/042027 A (CHIRON CORPORATION) 20 April 2006 (2006-04-20) page 7, paragraph 43 - page 9, paragraph 50 page 11, paragraph 60 page 14, paragraph 70 - page 18, paragraph 79 page 19, paragraph 87 - page 27, paragraph 110 page 30, paragraph 121 - page 70, paragraph 280; example 5 Tables 1-3; 7, 16; Antigen GAS 25; SEQ ID NOs:296, 297 claims ----- -/--	1-15

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 September 2008	10/09/2008

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Montero Lopez, B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2007/022838

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93/05156 A (BECKMAN INSTRUMENTS, INC.) 18 March 1993 (1993-03-18) page 2, line 3 - line 16 page 10, line 25 - page 11, line 6; example 8; sequence 2	1,2
A	US 5 700 648 A (KEHOE MICHAEL [GB] ET AL) 23 December 1997 (1997-12-23) column 2, line 8 - line 31 column 2, line 44 - column 3, line 8 column 8, line 25 - column 9, line 12	1-15
A	GB 2 233 977 A (KEHOE MICHAEL; PINKNEY MICHAEL) 23 January 1991 (1991-01-23) the whole document	1-15
A	US 5 354 846 A (KEHOE MICHAEL [GB]) 11 October 1994 (1994-10-11) column 2, line 23 - line 40 column 3, line 46 - column 4, line 32 page 6, line 14 - line 38; figure 1	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2007/022838

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
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			CA	2583803 A1	20-04-2006
			EP	1807446 A2	18-07-2007
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			AU	661028 B2	13-07-1995
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			DE	69222749 T2	26-02-1998
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			JP	3595840 B2	02-12-2004
			US	5378620 A	03-01-1995
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GB 2233977	A	23-01-1991	NONE		
US 5354846	A	11-10-1994	NONE		